

4,499,073

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adjusted to 4.0 by addition of 1 N HCl. Approximately 40 l. of HCl was added at a rate of less than one liter per minute with thorough mixing. The Fraction III filtrate was then metered into an ultrafiltration system. Ultrafiltration and diafiltration were used to reduce the alcohol concentration as rapidly as possible while holding the product temperature less than 10° C. Cold distilled water was used to maintain a constant volume of approximately 350 liters. Flux rates as high as 20 l. per minute were observed. When all the Fraction III filtrate had been concentrated to about 5% protein and the product alcohol concentration had been reduced to less than 8%, seven volume exchanges were performed using cold distilled water. The product temperature was permitted to drift as high as 20° C. The immune serum globulin solution was then concentrated to 8% protein and drained from the ultrafiltration system; 120 l. of 8% immune serum globulin was recovered in a clear "water-like" state. This material had an ionic strength of 0.001 (as determined by calculation) and a pH of 4.2. An aliquot of this material was made tonic with 10% maltose at 5% protein. This was filled into 250 ml bottles (60) for stability and other testing. Initial high pressure liquid chromatography (HPLC) results indicated a monomer level greater than 99%. This lot passed all typical testing for IGIV (Table 1). Several containers were stored at room temperature and after six months, HPLC results indicate the monomer content was still greater than 99%.

TABLE 1

HPLC Monomer (99.1%) Dimer (0.9%) Trimer (0) Void (0)	
Anticomplement Activity	3 mg protein per C'H50 unit
PKA	11% of reference
Buffer Capacity	16.24 meq./l.
Ultra-centrifuge	6.6S 90.8%
	9.8S 9.2%
Nephelometer	1.5 NTU

A similar aliquot was made tonic by addition of glycine to a concentration of 0.2 M.

## EXAMPLE 2

An aliquot (6 l.) of the 120 l. of 8% immune serum globulin prepared in Example 1 was treated with 1 N HCl to obtain a pH of 4.0 and lyophilized.

Water for injection was added to this material to obtain a 5% protein concentration. The reconstituted material exhibited the following characteristics:

TABLE 2

HPLC Monomer (98.5%) Dimer (1.5%) Trimer (0) Void (0)	
Anticomplement Activity	3 mg protein per C'H50 unit

What is claimed is:

1. A pharmaceutical composition comprising a solution, in a pharmaceutically acceptable aqueous carrier, of an immune serum globulin that is substantially free from chemical modification, said solution having a

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physiologically acceptable tonicity and an ionic strength such that the solution at 5 percent protein concentration has a nephelometric reading less than 15 NTU and a pH which ranges from about 3.5 to 5.0 to maintain, without significant change during storage at room temperature for extended periods, the monomer content at greater than about 90% and the actual anticomplement activity of the immune serum globulin at a level greater than about 2 mg protein/C'H50 unit wherein one C'H50 unit is defined as the amount of protein capable of inactivating 50% of complement in an optionally titrated complement and hemolysin system such that the composition is intravenously administrable and has a buffer capacity which causes very little disruption, if any, of the physiological pH when compared with the administration of an immune serum globulin at equivalent pH but essentially buffered.

2. The composition of claim 1 having an ionic strength (I/2) less than about 0.001.

3. The composition of claim 1 wherein the immune serum globulin is hyperimmune serum globulin.

4. The composition of claim 3 wherein the immune serum globulin is tetanus hyperimmune serum globulin.

5. The composition of claim 3 wherein the immune serum globulin is rabies hyperimmune serum globulin.

6. A method for rendering immune serum globulin intravenously injectable, which comprises

(a) forming an aqueous solution of immune serum globulin that is substantially free from chemical modification,

(b) adjusting the pH to about 3.5 to 5.0 and ionic strength of the solution such that the solution at 5% protein concentration has a nephelometric reading less than 15 NTU to maintain, without significant change during storage at room temperature for extended periods, the monomer content at greater than about 90% and the actual anticomplement activity of the immune serum globulin at a level greater than about 2 mg protein/C'H50 unit wherein one C'H50 unit is defined as the amount of protein capable of inactivating 50% of complement in an optionally titrated complement and hemolysin system such that the composition is intravenously injectable and has a buffer capacity which causes very little disruption, if any, of the physiological pH when compared with the administration of an immune serum globulin at equivalent pH but essentially buffered,

(c) adjusting the tonicity of the solution to a physiologically acceptable level by addition of an agent selected from the group consisting of amino acids, carbohydrates, and sugar alcohols, and mixtures thereof, and

(d) sterilizing the solution.

7. A composition comprising the product of claim 6.

\* \* \* \* \*

# United States Patent [19]

Lundblad et al.

[11] 4,186,192

[45] Jan. 29, 1980

[54] STABILIZED IMMUNE SERUM GLOBULIN

[75] Inventors: John L. Lundblad, El Cerrito; Willis L. Warner, San Rafael; Peter M. Fernandes, Concord, all of Calif.

[73] Assignee: Cutter Laboratories, Inc., Berkeley, Calif.

[21] Appl. No.: 970,686

[22] Filed: Dec. 18, 1978

[51] Int. Cl.<sup>2</sup> ..... A61K 39/00; A61K 35/14; A61K 37/00

[52] U.S. Cl. .... 424/85; 424/101; 424/177

[58] Field of Search ..... 424/177, 101, 85

[56] References Cited

## U.S. PATENT DOCUMENTS

4,089,944 5/1978 Thomas ..... 424/101

## OTHER PUBLICATIONS

Chem. Abstr. 80, 1974, 124750r.

Chem. Abstr. 77, 1972, 52306p.

Chem. Abstr. 87, 1977, 150657c, 150658f, 150659g.

Primary Examiner—Delbert R. Phillips

Attorney, Agent, or Firm—James A. Giblin; Robert E. Allen; Bertram Bradley

[57] ABSTRACT

Composition comprising an aqueous solution of immune serum globulin and maltose, the amount of maltose being sufficient to inhibit "shedding" of the globulin with time.

.. 20 Claims, 2 Drawing Figures

U.S. Patent

Jan. 29, 1980

4,186,192

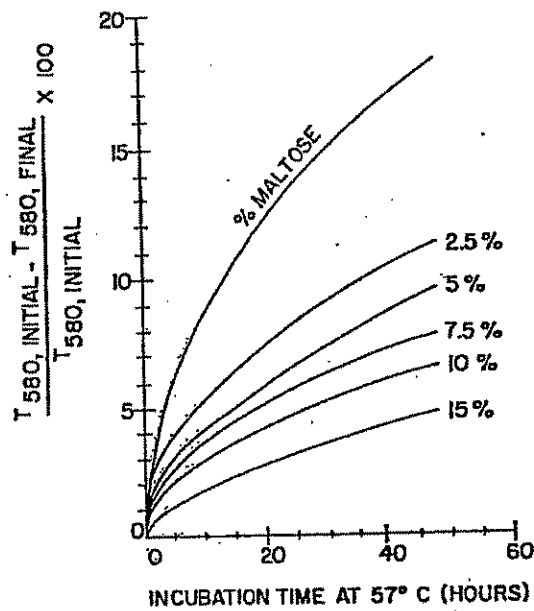


FIG. 1

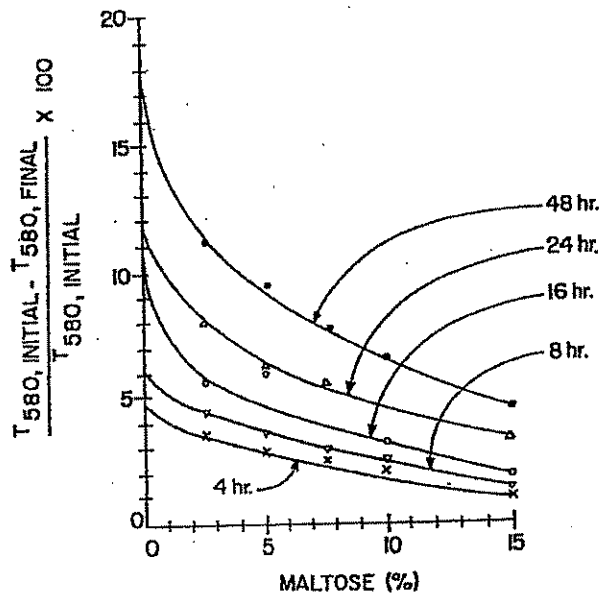


FIG. 2

4,186,192

## STABILIZED IMMUNE SERUM GLOBULIN

## BACKGROUND OF THE INVENTION

## 1. Field

This disclosure is concerned generally with immune serum globulin (ISG) preparations and specifically with a highly stabilized immune serum globulin solution.

## 2. Prior Art

It is well known that many plasma protein preparations intended for administration to humans or animals require stabilizers to prevent denaturation or other alteration prior to use. Instability of protein preparations is particularly observed as a function of concentration. Some, like immune serum globulin preparations, are particularly unstable in relatively dilute solutions (e.g. under 15 wt % concentration) of the protein. This instability, which may be manifest by the formation of insoluble particles ("shedding"), is often increased when storage of the protein preparation is at temperatures higher than refrigerator temperature (about room temperature or higher).

Various additives for stabilizing protein preparations have been used with varying degrees of success. For example, increasing the concentration of the protein or adding another protein such as albumin has enhanced stability in some cases. Unfortunately, however, such preparations may not always be acceptable for therapeutic purposes. It is known that amino acids are useful in stabilizing some protein preparations and degraded gelatin is commonly used as a stabilizer, especially in European countries. In considering an appropriate stabilizer, consideration should be given to such factors as lack of antigenicity (possible with gelatin), effect on osmolality of the final solution, biological activity of the specific proteins being stabilized, and the availability and cost of the stabilizer.

Various carbohydrates have been used to stabilize, facilitate processing, and/or enhance the solubility of certain biologically active protein preparations. For example, U.S. Pat. No. 2,826,533, to Fowell discloses the use of dextrose to increase the solubility of a fibrinogen preparation. U.S. Pat. No. 4,089,944 to Thomas discloses the use of a variety of carbohydrates (e.g. dextrose, mannose, galactose, fructose, lactose, sucrose, and maltose) to increase the solubility of an AHF-fibrinogen composition. The stabilization of plasma with invert sugar is disclosed in U.S. Pat. No. 3,057,781 to Mace et al.

Although dextrose has been added to immune serum globulin (e.g. Intraglobin ®), a modified immune serum globulin) to enhance stability and/or solubility, it has been found that, with time, the globulins in commercially available samples tend to aggregate, thereby increasing the optical density and resulting in a phenomenon commonly referred to as "shedding". As used herein, "shedding" means a visible precipitation of protein molecules. It is thought that shedding is caused by aggregation of the globulin molecules, rendering the molecules insoluble, especially in dilute solution. However, it should be noted that the exact nature of shedding is not fully understood. Shedding is undesirable since it is visually observable and indicates the possibility of inactivation or denaturation of the shed protein and, hence, lessens the effective amount of globulin available. In addition a solution of globulin having shed-

ded protein is unsatisfactory as a product in terms of visual appearance.

Another disadvantage associated with the use of known sugars as stabilizers in protein solutions is the fact that some sugar solutions tend to brown on heating. In addition, in some instances, it may be desirable to avoid the use of rapidly assimilated sugars such as dextrose in products intended for human use, especially for use in diabetic patients.

Quite surprisingly, we have developed a stabilized immune serum globulin preparation substantially free of shedding in dilute concentration over prolonged periods of time which utilizes a common, relatively inert sugar which, in general, can be used in amounts sufficient to assure a pharmaceutically acceptable isotonic globulin solution. This sugar can be used to stabilize immune serum globulin (ISG) preparations suitable for intramuscular administration (IMGG) or specially treated to render it suitable for intravenous administration (IVGG). Details of our stabilized preparations are disclosed herein.

## SUMMARY OF THE INVENTION

Our stabilized immune serum globulin preparation comprises an aqueous solution of a therapeutically effective amount of molecules of immune serum globulin (ISG) and maltose, the amount of maltose being sufficient to inhibit substantially the shedding of the globulin molecules with time. In one preferred embodiment, the preparation comprises a sterile, pharmaceutically acceptable solution of about 16.5 weight percent IMGG or 5 to 10 weight percent IVGG and maltose, the maltose being present in an amount to assure pharmaceutically acceptable isotonicity of the globulin solution. In another preferred embodiment the globulin solution includes about 2.5 to about 18 wt. % (preferably about 5 to 15 wt. %) of the maltose, a very preferred amount of maltose being about 10 weight percent. In yet another preferred embodiment the solution includes about 10 weight percent maltose and an amount of glycine, preferably about 0.1 M glycine.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a graph illustrating the stability of an IVGG solution as a function of time and maltose concentration at 57° C.

FIG. 2 is a graph further illustrating the beneficial effects of increasing amounts of maltose on the stability of an IVGG solution over a period of time at 57° C.

## SPECIFIC EMBODIMENTS

The stabilizing agent of this disclosure is especially useful for lower concentration solutions of ISG (IMGG or IVGG) where, because of a relatively small amount of protein, shedding is more likely and apparent.

The immune serum globulin (IMGG) or immune serum globulin modified for intravenous injection (IVGG) are well known and can be prepared by known means. For example the IMGG (frequently available as a 16.5 wt. % solution) is commonly prepared via Cohn fractionation (see, Cohn et al, J. Am. Chem. Soc., 68, 459-475 (1946); Oncley et al, J. Am. Chem. Soc. 71, 541-550 (1949)). Examples of preparing an IVGG are disclosed in U.S. Pat. No. 3,903,262 to Pappenhagen et al. The specific disclosures of the above publications are incorporated herein by reference thereto.

The maltose used to stabilize the aqueous globulin solutions is described in detail in, for example, The

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Merck Index, Ninth Edition, Merck & Co., Inc., Rahway, N.J. (1976). The disaccharide maltose is readily available in pure form and has good stability in aqueous solutions up to 20 weight percent which can be autoclaved without browning of the solution. Physiologically in small quantities it is practically inert. When administered intravenously, it is partially converted to glucose by the specific enzyme maltase found in many tissue sites in most animal species, including humans.

Some administered maltose is rapidly excreted by the human kidney unchanged without significant diuresis. The conversion to glucose is gradual and frequently undetectable when plasma glucose is serially measured; there is no apparent increase in circulating insulin levels. No adverse reactions have been reported, even after the administration of up to 200 grams of maltose in four hours as a 10% aqueous solution. Since maltose is a disaccharide, a 10% solution is approximately isotonic in humans. As used herein, the expression pharmaceutically acceptable isotonicity refers to that range of osmolality in a pharmaceutical solution which, in general, will not result in significant local adverse effects (e.g., vessel wall irritation). Although the amount of maltose needed to assure an osmolality within this range will vary depending on such factors as amino acid concentration, salt concentration, etc., in general, we prefer using about 5 to 15 weight percent maltose in the IMGG or IVGG solution, preferably about 10 weight percent.

More important however in determining the proper amount of maltose is the need to avoid shedding in the IMGG or IVGG solution with time. As noted above, shedding is a term referring to a noticeable precipitation of the globulin molecules, especially prevalent as the concentration of the protein solution is decreased. The phenomenon of shedding can be accurately observed and recorded by noting the spectrophotometric transmittance of light at 580 nm of a given solution. Differences in transmittance with and without varying amounts of the maltose provide a means of determining the degree of shedding over a period of time at a given temperature. Shedding can also be noted visually. As used herein, the substantial inhibition of shedding means a change in transmittance at 580 of less than 10%, preferably less than 5% when the solution is heated for 24 hours at 57° C. It can be appreciated that shedding will occur more slowly at lower temperatures.

In preliminary studies directed toward understanding the nature of the shedding phenomenon, it was found that variation in the final container pH of a 5% IVGG solution in the allowable range, pH 6.4 to 7.2, had no effect on the degree of shedding. Further studies indicated that non-carbohydrate addition such as NaCl or glycine alone were not effective in stabilizing the protein solution for more than a very short period. A combination of NaCl and glycine was also not effective.

From the above studies, it becomes apparent that the accelerated precipitation test offered a simple and rapid test of the ability of IMGG or IVGG to resist instability due to storage under adverse conditions, as well as an indication of the extent to which the character of the molecule had been preserved.

A variety of carbohydrates at varying concentrations were investigated (i.e. dextrose, fructose, mannitol, sorbitol and maltose) as potential stabilizers for the protein solution. In all cases, stability appeared to be directly proportional to sugar concentration. In all cases the final container material was more clear using

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the carbohydrates than preparations which included glycine and NaCl.

It was found that maltose was the best overall stabilizer for clinical acceptability. For example, dextrose can cause undesirable hyperglycemia; mannitol is a diuretic at effective concentrations; and sorbitol and fructose have adverse effects in acid-base balance.

In a series of experiments in which the amount of maltose was increased from 5% to 18%, it was also found that the addition of glycine significantly improved the clarity of a 5% IVGG solution. Thus, although the maltose addition results in a stable product, substantially free from shedding, in a very preferred embodiment glycine is included (e.g. about 0.1 M glycine for a 5% IVGG solution). The data summarizing the effects of maltose, glycine, and combination of the two on IVGG clarity are summarized in the table below.

Table I

Effect of Maltose and Maltose-Glycine on IVGG Clarity			
Additive	Initial T <sub>580</sub> %	Final T <sub>580</sub> %	% Change
5% Maltose	99.05	95.69	3.39
7% Maltose	99.25	96.81	2.76
10% Maltose	99.35	97.24	2.13
13% Maltose	99.25	97.87	1.39
18% Maltose	99.45	98.07	1.39
5% Maltose, 0.1M Glycine	99.15	97.29	1.88
7% Maltose, 0.1M Glycine	99.35	97.58	1.78
10% Maltose, 0.1M Glycine	99.35	98.22	1.14
13% Maltose, 0.1M Glycine	99.50	98.76	0.75
18% Maltose, 0.1M Glycine	99.60	98.76	0.85
5% Maltose, 0.3M Glycine	99.15	98.07	1.09
7% Maltose, 0.3M Glycine	99.35	98.66	0.70
10% Maltose, 0.3M Glycine	99.45	98.96	0.50
13% Maltose, 0.3M Glycine	99.45	99.05	0.40
18% Maltose, 0.3M Glycine	99.65	99.25	0.40

It should be pointed out that maltose is an especially preferred stabilizer since it is a relatively harmless sugar and sufficient data are available on its toxicological and clinical effects. Glycine in combination with maltose does improve clarity and is a commonly used stabilizer for protein solutions. Because of this and since glycine also contributes to the osmolality of the product, it was found that a preferred solution should contain only 0.1 M glycine and 10% maltose. Osmolality values for some maltose-glycine formulations which supported the above findings are summarized in the table below.

Table II

Effect of Additive on Osmolality of 5% IVGG Solution	
Additive plus 5% IVGG	Osmolality, mOsm/kg
10% Maltose, 0.3M Glycine	466
10% Maltose, 0.1M Glycine	366
10% Maltose	300
0.3M Glycine, 0.45% NaCl	413

The beneficial effects of increasing maltose concentrations on the long term stability (accelerated studies using the temperature of 57° C.) are summarized in FIGS. 1 and 2 where the change in absorbance at 580 as functions of maltose concentrations and time at 57° C. is dramatically illustrated. From FIG. 1 it can be seen that shedding was substantially inhibited with as little as about 2.5% maltose.

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## EXAMPLE

The preparation of the product of this disclosure in a very preferred embodiment using the best methodology known to date is illustrated for a specific 5% IVGG solution below. It can be appreciated, however, that those skilled in the art in view of this disclosure will now be able to prepare readily any stabilized ISG or IVGG solution. Our illustrative stabilized product was prepared as follows:

15 grams of Cohn Fraction II paste are suspended in a 0.45% sodium chloride solution at 0-5° C. such that the concentration of protein is  $5 \pm 0.2\%$ .

The solution (approximately 100 ml) is warmed to 22-25° C. and the pH adjusted to  $8.1 \pm 0.1$  with 1 N sodium hydroxide. 0.387 g/L of solution of Dithiothreitol (Aldrich Chemical Company, Milwaukee, Wis.) is added and the ensuing chemical reduction is allowed to proceed for 15 minutes. Iodoacetamide (Aldrich Chemical Company) is then added at a concentration of 1.018 g/L of solution and the alkylation process is allowed to occur for 60 minutes. The pH of the solution is maintained at  $8.1 \pm 0.1$  by addition of 1 M sodium hydroxide or 0.8 M sodium acetate buffer.

The pH is reduced to 6.8 on completion of the protein modification process and the solution subjected to extensive diafiltration for removal of residual reagents. This process consists of diafiltering the protein solution for at least a total of seven volume replacements against the following solutions:

0.45% sodium chloride for the first three replacements.

Water for injection for a minimum of four replacements.

The solution is clarified using a non-asbestos containing filter and maltose and glycine added such that the final composition contains:

Protein—5%

Maltose—10%

Glycine—0.75% (0.1 M)

The pH of the solution is adjusted to  $6.8 \pm 0.1$  and the material is sterile filtered through a 0.2 micron filter and filled into appropriate final containers.

Inasmuch as the above described disclosure is subject to numerous variations which are or will become apparent to those skilled in the art, it is intended that the disclosed examples be construed as illustrative only and that the described invention be limited only by the following claims.

We claim:

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1. A stabilized immune serum globulin preparation comprising an aqueous solution of a therapeutically effective amount of molecules of ISG and maltose, the amount of maltose being sufficient to inhibit shedding of the globulin over prolonged periods of time.

2. The preparation of claim 1 wherein the amount of maltose ranges from about 2.5 to about 18 weight percent.

3. The preparation of claim 2 wherein the amount of maltose ranges from about 5 to about 15 weight percent.

4. The preparation of claim 3 wherein the amount of maltose is about 10 weight percent.

5. The preparation of claim 1 wherein the solution includes glycine.

6. The preparation of claim 5 wherein the glycine is present in a concentration of about 0.1 M.

7. The preparation of claim 1 wherein the globulin present is IMGG, and the amount of the globulin is about 16.5 wt. %.

8. The preparation of claim 1 wherein the globulin is IVGG, and the amount of the globulin is about 5 wt. %.

9. The preparation of claim 1 wherein amount of maltose is sufficient to assure pharmaceutically acceptable osmolality.

10. The preparation of claim 9 wherein the osmolality of the solution ranges from about 170 to 600 mOsm/kg.

11. The preparation of claim 10 wherein the osmolality is about 260 to 500 mOsm/kg.

12. A sterile, pharmaceutically acceptable preparation comprising an aqueous solution of molecules of an immune serum globulin, the globulin having been modified for intravenous administration, and maltose, the maltose being present in an amount sufficient to inhibit substantial shedding of the globulin molecules.

13. The preparation of claim 12 wherein the amount of maltose is sufficient to inhibit visually detectable shedding.

14. The preparation of claim 12 wherein the amount of maltose ranges from about 2.5 to about 18 wt. %.

15. The preparation of claim 14 wherein the amount of maltose ranges from about 5 to about 15 wt. %.

16. The preparation of claim 15 wherein the amount of maltose is about 10 wt. %.

17. The preparation of claim 16 wherein the aqueous solution includes about 5-weight percent of the globulin.

18. The preparation of claim 12 wherein the solution includes glycine.

19. The preparation of claim 18 wherein the glycine concentration is about 0.1 M.

20. The preparation of claim 12 wherein the solution has a pharmaceutically acceptable osmolality.

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,186,192

DATED : January 29, 1980

INVENTOR(S) : PETER M. FERNANDES, JOHN LUNDBLAD, WILLIS L. WARNER

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

On the information-bearing front page, the inventors names should appear as follows:

Inventors: Peter M. Fernandes, Concord;  
John L. Lundblad, El Cerrito;  
Willis L. Warner, San Rafael,  
all of California.

Signed and Sealed this

Twentieth Day of May 1980

[SEAL]

Attest:

SIDNEY A. DIAMOND

Attesting Officer

Commissioner of Patents and Trademarks



US005159064A

**United States Patent** [19][11] Patent Number: **5,159,064****Mitra et al.**[45] Date of Patent: \* **Oct. 27, 1992**[54] **PREPARATION OF VIRUS-FREE ANTIBODIES**[75] Inventors: **Gautam Mitra, Kensington; Milton M. Mozen, Berkeley, both of Calif.**[73] Assignee: **Miles Inc., Elkhart, Ind.**[\*] Notice: **The portion of the term of this patent subsequent to Aug. 9, 2005 has been disclaimed.**[21] Appl. No.: **471,571**[22] Filed: **Jan. 29, 1990****Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 192,044, May 9, 1988, Pat. No. 4,948,877, which is a continuation of Ser. No. 849,612, Apr. 4, 1986, Pat. No. 4,762,714.

[51] Int. Cl.<sup>5</sup> ..... **C07K 15/06; C12N 9/00; A61K 35/14; A61K 39/395**[52] U.S. Cl. .... **530/388.1; 424/85.8; 424/530; 435/236; 514/2**[58] Field of Search ..... **530/387, 808, 388.1; 424/85.8, 530; 435/70.21, 236; 514/2**[56] **References Cited****U.S. PATENT DOCUMENTS**

2,897,123	7/1959	Singher	424/101
4,396,608	8/1983	Tenold	424/101
4,440,679	4/1984	Fernandes et al.	424/101
4,640,834	2/1987	Eibl et al.	424/101
4,762,714	8/1988	Mitra et al.	424/101
4,948,877	8/1990	Mitra et al.	530/387

*Primary Examiner—Jacqueline Stone**Attorney, Agent, or Firm—James A. Giblin*[57] **ABSTRACT**

Antibodies, including monoclonal antibodies (Mabs), can be made substantially free of infectious viruses by storing them in a liquid state at conditions of pH, temperature and time sufficient to inactivate substantially all infectious viruses. Preferred inactivation methods involve use of a pH equal to or less than about 4.0 at a temperature of at least about 5° C. for at least about 16 hours.

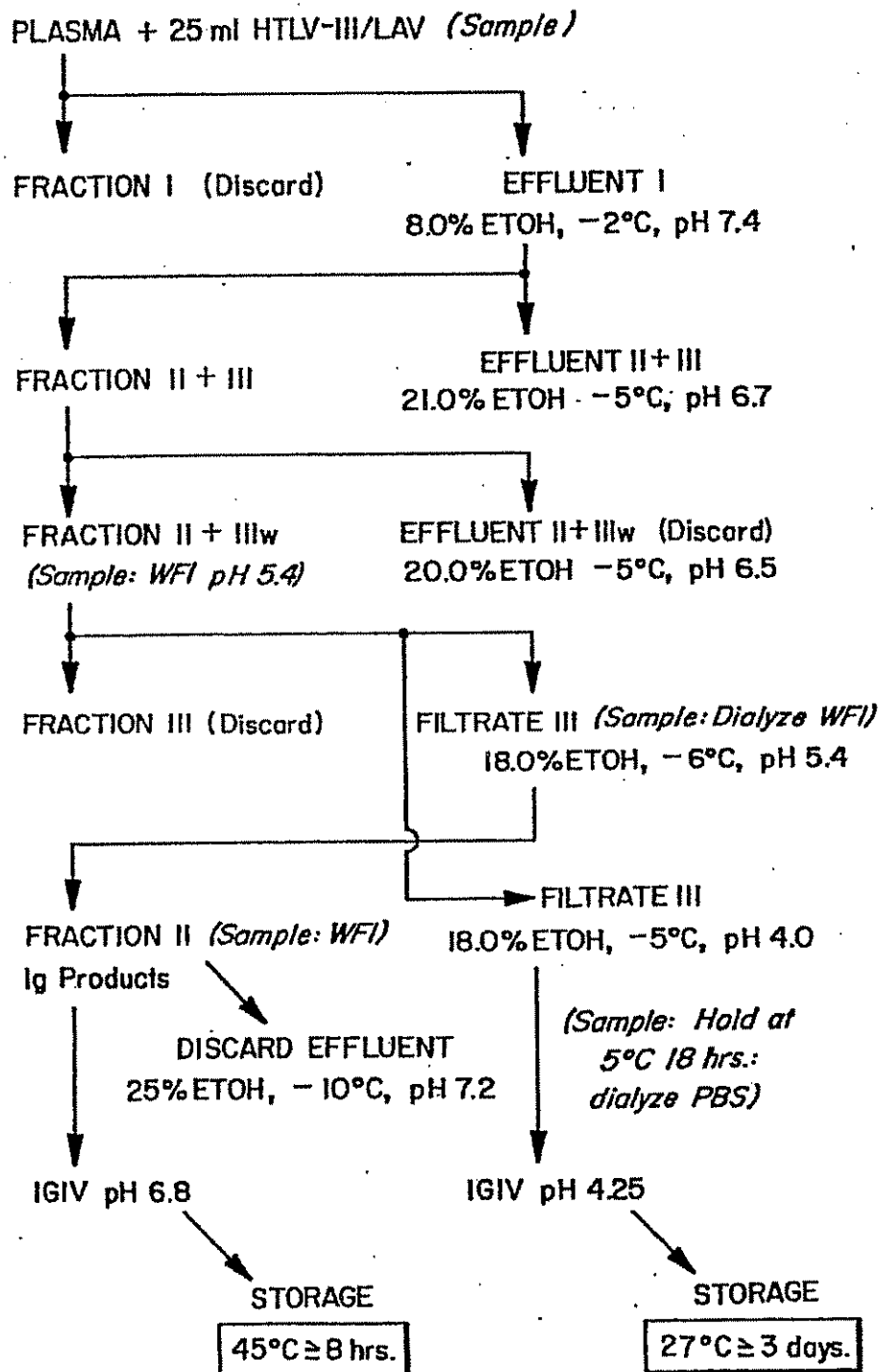
**4 Claims, 1 Drawing Sheet**

U.S. Patent

Oct. 27, 1992

5,159,064

## HTLV-III/LAV Plasma Fractionation



5,159,064

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## PREPARATION OF VIRUS-FREE ANTIBODIES

This is a continuation-in-part of Ser. No. 07/192,044 filed on May 9, 1988, U.S. Pat. No. 4,948,877, which is a continuation of patent application Ser. No. 06/849,612 filed Apr. 4, 1986, now U.S. Pat. No. 4,762,714.

### BACKGROUND OF THE INVENTION

#### 1. Field

This disclosure is concerned generally with the inactivation of viruses and retroviruses in immune serum globulin (ISG) and specifically with the inactivation of such viruses and retroviruses as the LAV strain of an AIDS virus in ISG intended for intravenous (IV) administration.

#### 2. Prior Art

Therapeutic and prophylactic ISG preparations are well known and have been available for many years. ISG is presently obtained in commercial quantities using variations of a blood plasma fractionation technique developed by Cohn et al in the 1940's. Although ISG has been administered intramuscularly (IM) and more recently intravenously (IV), the latter route of administration provides numerous advantages and has gained acceptance as the preferred route of administration.

Initial attempts to render an ISG safe and effective for IV administration (IVIG) focused on eliminating its anticomplement activity. In one approach, for example, this involved chemically modifying the ISG (see U.S. Pat. No. 3,903,262 to Pappenhagen et al). More recently, the ISG has been made suitable for IV administration through careful pH and ionic strength control (see U.S. Pat. No. 4,396,608 and 4,499,073 both to Tenold). It is also known that IVIG preparations can be stabilized with carbohydrates such as maltose (see U.S. Pat. No. 4,186,192 to Fernandes et al). ISG preparations can be further purified using a variety of techniques (see, for example, U.S. Pat. No. 4,272,521 to Zuffi). Various ISG preparations having a relatively high titer to a given antigen are also well known (e.g. tetanus, hepatitis, Rho factor, etc.).

Although ISG products (both IMIG and IVIG) have been considered generally safe, there has been a growing need to assure patients that ISG products do not transmit active viruses such as those associated with hepatitis or, more recently, retroviruses such as that associated with Acquired Immune Deficiency Syndrome (AIDS). The present disclosure is based on work done to address such needs.

Antibodies to a retrovirus associated with the AIDS have been detected in human hepatitis B immunoglobulin (HBIG) (see Tedder, R. S. et al, Safety of immunoglobulin preparation containing anti-HTLV-III, *Lancet* 1985;1:815) as well as in other commercial lots of immunoglobulins (see Gocke, D. J. et al, HTLV-III antibody in commercial immunoglobulin, *Lancet* 1986;1:37-8). This observation raised the possibility that immunoglobulin product transmit infectious virus. This concern was heightened by recent reports of non A, non B (NANB) hepatitis in immunodeficient patients who had received infusions of intravenous immunoglobulins prepared from Cohn fraction II (see Webster, A. D. B. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, *Lancet* 1986;1:322, and Ochs, H. D. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, *Lancet* 1986;1:322-23).

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Based on the above findings, we decided to determine the ability of retroviruses to withstand the various procedures employed in immunoglobulin preparations as well as other procedures. For these experiments, two prototype retroviruses were used: the mouse xenotropic type C retrovirus and the LAV strain of the AIDS retrovirus. Surprisingly, we found that the model retroviruses could be inactivated in ISG prepared by a known fractionation processing technique if that technique is followed by storage at controlled conditions of pH, temperature and time. Details of our method are described below.

### SUMMARY OF THE INVENTION

We have found that ISG preparations can be made substantially free of retrovirus such as a LAV strain associated with AIDS by preparing the ISG from pooled plasma using a known processing technique (i.e. Cohn-Oncley cold ethanol process, using at least about 18% ethanol v/v at pH 5.4), followed by storage of the ISG at a pH of less than 5.4, a temperature of at least about 27° C., or at a pH of 6.8 at a temperature of at least about 45° C. for periods sufficient to assure retrovirus inactivation. In preferred embodiments, our ISG preparation is stabilized with a carbohydrate (e.g. maltose) and in a 5% wt./vol. liquid (aqueous) form. It is intended for IV use and is made substantially free (less than 10 infectious virus particles) of the LAV strain of retrovirus associated with AIDS by processing pooled human plasma using the Cohn-Oncley cold ethanol process (about 18% ethanol, pH  $\geq$  5.4) to obtain ISG followed by storage of the ISG at a pH of about 4.25 for at least about 21 days at a temperature about 27° C. In another embodiment, the ISG may be stored at pH 6.8 for about 45° C., for at least 8 hours to assure the retrovirus inactivation.

### BRIEF DESCRIPTION OF THE FIGURE

The FIG. illustrates a flow chart of the steps used in our Cohn-Oncley cold ethanol fractionation of human plasma, including the novel storage conditions disclosed herein.

### SPECIFIC EMBODIMENTS

#### Materials and Methods

The mouse xenotropic type C retrovirus recovered from a New Zealand Black mouse kidney was grown to high titer in mink lung cells (Varnier, O. E. et al, Murine xenotropic type C viruses. V. Biological and structural differences among three cloned retroviruses isolated from kidney cells from one NZB mouse, *Virology* 1984;132:79-94). Detection was based on a focus assay in mink S+L-cells in which each infectious particle scores as an area of cell transformation (Peeples, P. T., An in vitro focus induction assay for xenotropic murine leukemia virus, feline leukemia virus C, and the feline Primate viruses RI-114/CCC/M-7, *Virology* 1975;67:288-91). Virus titer was also determined by the induction in cells of the viral core structural protein (page 30) measured by immunofluorescence (see Levy, J. A., Xenotropic type C viruses, *Current Topics Microbiol. Immunol.* 1978;79:111-212). The use of these assays for detection of mouse C virus in spiking experiments with plasma fractions has previously been described by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, *Lancet* 1984;iii:722-723 and Levy, J. A. et

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al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, *Lancet* 1985;ii:1456-1457).

LAV was cultured and obtained from the Centers for Disease Control (CDC) in Atlanta, Ga. Its detection was based on a sandwich enzyme-linked immunoassay (ELISA) previously described (see McDougal, J. S. et al, Immunoassay for the detection and quantitation of infectious human retrovirus, lymphadenopathy-associated virus [LAV], *J. Immunol. Methods* 1985;76:171-183).

Human plasma samples were spiked with retroviral preparations and fractionated according to classical Cohn-Oncley cold ethanol procedures (see Cohn, E. J. et al, Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of protein and lipoprotein components of biological tissues and fluids, *J. Am. Chem. Soc.* 1946;68:459-75 and Oncley, J. L. et al, The separation of the antibodies, isoagglutinins, prothrombin, plasminogen, and beta-1 lipoprotein into subfractions of human plasma, *J. Am. Chem. Soc.* 1949;71: 541-50). The fractionation was accomplished through selective precipitations in the cold at various ethanol concentrations and pH values: fraction I at 8% ethanol,  $-2^{\circ}\text{C}$ , pH 7.4; fraction II + III at 21% ethanol,  $-5^{\circ}\text{C}$ , pH 6.7; fraction II + IIIw at 20% ethanol,  $-5^{\circ}\text{C}$ , pH 6.5; fraction III at 18%

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Twenty ml of virus concentrate was added to 200 ml of plasma for the fractionation studies described. The fractionation methods and viral assays are described in the text. Total IP = total infectious particles. Total  $\text{ID}_{50} = \text{ID}_{50}$  (reciprocal of dilution at which 50% of the cultures are positive)  $\times$  volume.

From plasma to fraction II + IIIw, no more than a 10-fold reduction of virus titer was observed. Preparation of filtrate III from fraction II + IIIw resulted in an approximately 10,000-fold reduction of the mouse type C retrovirus and 10-fold reduction in LAV. Due to dilution, ethanol concentration decreased from 20% v/v to 18% v/v across this fractionation step and the pH was reduced from 6.50 to 5.40. Fraction II precipitation from filtrate III resulted in  $>1,000$ -fold reduction in titer of both the infectious mouse and human retroviruses. During this fractionation step, the pH was raised to 7.25 and the ethanol concentration increased to 25%. The 1,000-fold loss of virus infectivity primarily results from virus inactivation (not fractionation) since after extensive dialysis, no infectious virus was measurable in the supernatant corresponding to fraction II (data not shown).

In studying more precisely the effect of pH and temperature on retrovirus inactivation with 18% ethanol, we mixed a quantity of the mouse retrovirus with filtrate III. See Table 2.

TABLE 2

Effect of pH and Temperature on Mouse Type C Retrovirus added to Filtrate III (18% Ethanol)						
Sample	Temperature $-5^{\circ}\text{C}$ .			Sample	Temperature $22^{\circ}\text{C}$ .	
	(a) pH 5.4 (Total IP)	(b) pH 4.7 (Total IP)	(c) pH 4.0 (Total IP)		(d) pH 5.4 (Total IP)	(e) pH 4.0 (Total IP)
Virus alone	$6.9 \times 10^6$	$7.9 \times 10^6$	$7.9 \times 10^6$	Virus alone	$4.0 \times 10^8$	$5.0 \times 10^7$
Virus + Filtrate III	$2.9 \times 10^6$	$3.4 \times 10^5$	$6.5 \times 10^5$	Virus + Filtrate III	$2.2 \times 10^9$	$5.5 \times 10^5$
2 hours	$3.4 \times 10^5$	$6.6 \times 10^6$	$2.0 \times 10^5$	3 hours	$2.2 \times 10^8$	Non-detectable
4 hours	$6.7 \times 10^5$	$6.7 \times 10^5$	$2.6 \times 10^5$			
6 hours	$7.8 \times 10^5$	$6.6 \times 10^5$	$4.1 \times 10^5$			

ethanol,  $-6^{\circ}\text{C}$ , pH 5.4; and fraction II collected at 25% ethanol  $-10^{\circ}\text{C}$ , pH 7.2. Residual retroviral levels were determined across the fractionation steps. The pH (range 5.4-4.0) and temperature (range  $-5^{\circ}\text{C}$  to  $22^{\circ}\text{C}$ ) effects on virus infectivity in the presence of ethanol (approximately 18%) were determined with filtrate III. Final container liquid immunoglobulin preparations, in the absence of ethanol, were incubated with retrovirus concentrates at  $27^{\circ}\text{C}$  and  $45^{\circ}\text{C}$ ; virus infectivity was determined at different time periods.

## RESULTS

Infectivity of both the mouse C and AIDS retrovirus was not affected by the addition of these viruses to human plasma at  $\leq 5^{\circ}\text{C}$ . See Table 1.

TABLE 1

Effect of Immunoglobulin fractionation procedures on infectious retrovirus added to plasma		
Store	Mouse Type C (Total IP)	AIDS Virus LAV (Total $\text{ID}_{50}$ )
Virus alone	$2.0 \times 10^5$	$2.3 \times 10^5$
Virus + plasma ( $5^{\circ}\text{C}$ )	$2.3 \times 10^5$	$4.4 \times 10^5$
II + IIIw	$3.8 \times 10^7$	$4.8 \times 10^4$
Filtrate III	$1.6 \times 10^3$	$1.7 \times 10^3$
Fraction II	Non-detectable	Non-detectable

At  $-5^{\circ}\text{C}$ , no significant virucidal effect was seen in the pH range of 5.4-4.0 for up to 6 hours (2a, b, c). At  $22^{\circ}\text{C}$  (ambient), however, at pH 4.0  $>100,000$  infectious mouse retrovirus particles were inactivated by 3 hours (2e). In contrast, at pH 5.4 under similar conditions, no significant virucidal effect was seen (2d). Similarly,  $1.7 \times 10^3$  total  $\text{ID}_{50}$  of LAV that was in a filtrate III solution at pH 4.0 and held at  $+5^{\circ}\text{C}$  for 18 hours, was reduced in titer to non-detectable level (data not shown). It therefore appears that the presence of 18% ethanol in plasma fractions at pH 5.4 is not markedly virucidal for these viruses in the temperature range of  $-5^{\circ}\text{C}$  to  $22^{\circ}\text{C}$ . Only when the pH is lowered (pH 4.0) concomitant with a raise in temperature ( $\geq 5^{\circ}\text{C}$ ), significant virus inactivation observed. For LAV, the following conditions were sufficient for a 1,000-fold reduction in infectious virus: ethanol 18%, pH 4.0, temperature  $+5^{\circ}\text{C}$ , time 18 hours (data not shown). For the mouse type C retrovirus,  $>10,000$ -fold reduction was measured under similar treatment conditions. To determine the effect on AIDS virus of pH and temperature of the final product, final container liquid immunoglobulin preparations (protein concentration 5% w/v) were incubated with LAV (Table 3). At  $27^{\circ}\text{C}$ , between 103-104 of total  $\text{ID}_{50}$  were inactivated by 3 days for the immunoglobulin preparations of both pH 6.8 and pH 4.25. At  $45^{\circ}\text{C}$ ,  $>10,000$  infectious particles were

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inactivated within 8 hours with the pH 6.8 immunoglobulin preparation. The pH 4.25 immunoglobulin preparation was not tested at 45° C.

#### DISCUSSION

These experiments were conducted to evaluate the effect on infectious retroviruses of procedures used for immunoglobulin fractionation. The data are important in evaluating the possible risk of AIDS virus contamination of some Ig preparations. The mouse type C retrovirus was used as well as the LAV strain of AIDS virus, because the former can be grown to very high titer and therefore the effect of various procedures can be better evaluated. In addition, a focus assay for the mouse virus allows more precise quantitation.

Unlike the reported complement-mediated lyses of many retroviruses in human serum at 37° C. (see Welsh, R. M. et al, Human serum lyses RNA tumor viruses, *Nature* 1975;257:612-14), the AIDS virus in the cold (0°-5° C.) is not affected by this mechanism (see Banapour, B. et al, The AIDS-associated retrovirus is not sensitive to lyses or inactivation by human serum, *Virology* [in press] 1986). The reported virucidal effects of ethanol for LAV have been at ambient temperature (see Spire, B. et al, Inactivation of lymphadenopathy associated virus by chemical disinfectants, *Lancet* 1984;ii:899-901 and Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/lymphadenopathy associated virus, *J. Infect. Dis.* 1985;152:400-403), whereas the data reported here show that these virus inactivating effects are diminished in the presence of plasma at low temperatures (<5° C.). Enhanced inactivation at low pH is demonstrated which again is strongly dependent on temperature. This observation agrees with an earlier report (see Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/lymphadenopathy associated virus, *J. Infect. Dis.* 1985; 152:400-403) indicating increased inactivation of LAV inoculum at pH extremes.

Filtrate III with 18% ethanol at pH 5.4 and at a temperature of -5° C. was not significantly virucidal for retroviruses for extended periods of time. Hence, the 100,000-fold reduction of the mouse type C virus and a 100-fold reduction of LAV from plasma to filtrate III is probably primarily due to fractionation under the processing condition (ethanol range 0-20% v/v, pH range 7.4-5.4) employed at -5° C. The reduction difference between the mouse and the human virus reflects either a greater resistance of the AIDS virus to the processing conditions or a less quantitative assay for this virus. As noted above, the mouse virus can be grown up to high titers and its assay is very reproducible. Its usefulness for fractionation/inactivation studies has been previously reported by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, *Lancet* 1984;ii:722-723 and Levy, J. A. et al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, *Lancet* 1985;ii:1456-1457).

Ethanol concentration is increased to 25% v/v at pH 7.20 for the fraction II precipitation which results in a more than 1,000-fold inactivation of the mouse type C

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virus and LAV. Since the corresponding effluent was free of infectious virus, true inactivation at the 25% ethanol concentration is most likely involved. A recent report (see Piskiewicz, D. et al, Inactivation of HTLV-III/LAV during Plasma fractionation, *Lancet* 1985;ii:1188-89) had shown inactivation of  $10^{4.5}$  ID<sub>50</sub> of the AIDS retrovirus during the precipitation of I+II+III (ethanol 20% v/v, pH 6.9, temperature -5° C.) under conditions in which fraction II+III is precipitated together with fraction I. Our results which isolate these fractions separately do not show such complete LAV inactivation under similar conditions (Table I). In our study, the samples were extensively dialyzed in PBS prior to ID<sub>50</sub> assay. In the other report, a 1:10 dilution to a resultant residual ethanol concentration of 2% v/v was used in the assay. Furthermore, it is not possible from the other report to distinguish whether the virus titer was being determined in the precipitate or the supernatant following I+II+III precipitation; hence, a meaningful comparison between the two studies is difficult to make.

Greater than a 1,000-fold drop in AIDS virus infectivity did result after its incubation with purified liquid immunoglobulin preparations at 27° C. for 3 days; pH of the purified immunoglobulin preparations did not seem to have an appreciable effect. A higher incubation temperature (45° C.) demonstrated comparable titer reduction within 8 hours. A "worse case" estimate of 2,000 ID/ml of AIDS virus in large plasma pools has been reported (see Petricciani, J. C. et al, Case for concluding that heat-treated, licensed antithaemophilic factor is free from HTLV-III, *Lancet* 1985;ii:890-891). The yield of IgG could be as low as 50% of the amount present in plasma together with IgG concentration increase from approximately 1 gm/100 ml in plasma to 5 gm/100 ml in purified product. If the AIDS virus was concentrated without loss of infectivity along with IgG purification, the purified IgG would contain 2,000 ID/ml  $\times 10$  ( $2 \times 10^4$  ID/ml) Immunoglobulin purification processes must therefore be able to fractionate/inactivate  $2 \times 10^4$  ID/ml of AIDS virus.

No single step in the Cohn cold ethanol process can completely inactivate retroviruses. The effects of fractionation and inactivation taken together through the fractionation cascade could be quite large. LAV recovery from plasma to fraction II is reduced by at least 100,000-fold; pH adjustment to 4.0 at filtrate III (at +5° C.) is as effective for viral inactivation as precipitation of fraction II in the presence of 25% ethanol. An extra margin of safety is provided when the final preparation in liquid form is incubated at 27° C., since these experiments demonstrated that in liquid immunoglobulin preparations, a 1,000-10,000-fold reduction of LAV occurred within 3 days under these conditions. Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus. *N. Eng. J. Med* 1986; 314:386-87, have suggested that the long storage of liquid immune serum globulin preparations may contribute to their safety. The studies presented here experimentally validate that AIDS virus are indeed inactivated during liquid storage. See Table 3.

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TABLE 3

Effect of pH and Temperature on LAV added to Final Container Liquid Immunoglobulin Preparations				
Sample	Temperature 27° C.		Sample	pH 6.8 IgG (Total ID <sub>50</sub> )
	pH 6.8 IgG (Total ID <sub>50</sub> )	pH 4.25 IgG (Total ID <sub>50</sub> )		
Virus + IgG	1.65 × 10 <sup>4</sup>	3.69 × 10 <sup>5</sup>	Virus + IgG	1.65 × 10 <sup>4</sup>
3 days	Non-detectable	Non-detectable	1 hour	6.27 × 10 <sup>3</sup>
12 days	Non-detectable	Non-detectable	4 hours	1.65 × 10 <sup>3</sup>
24 days	Non-detectable	Non-detectable	8 hours	Non-detectable
			20 hours	Non-detectable

The chance for an infectious retrovirus to survive this fractionation as well as storage of the liquid final preparation, is therefore extremely small, if at all.

The fractionation/inactivation and final container incubation results reported here support the available clinical and epidemiological evidence that therapeutic immunoglobulins prepared by Cohn-Oncley cold ethanol process (≥ 18% v/v ethanol, pH ≤ 5.4 at filtrate III) do not transmit AIDS viruses particularly after storage at a pH of 4.25 at a temperature of 27° C. for about 3 days or at pH 6.8 at temperature of 45° C. for at least 8 hours. The conditions of the Cohn-Oncley process i.e., alcohol concentration, pH, temperature, do not in themselves inactivate AIDS virus as recently reported by Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus, N. Eng. J. Med. 1986;314:386-87. As described, their study was primarily geared towards determining inactivation, and no sequential fractionation was carried out with a virus spike. The present study, in contrast, mimics a true fractionation run and hence portrays a realistic virus carryover estimate involving the sum total of fractionation and inactivation:

It is important to emphasize that variations from classical Cohn approach need to be validated in terms of their virucidal and virus distribution potential since fractionation, ethanol concentration, pH, and temperature all play an important role in virus recovery. It is possible that total log reduction of different viruses could be different and hence it would be difficult to generalize these virus recovery results for other viruses.

#### FURTHER STUDIES

Further studies with monoclonal antibodies have confirmed that a low pH viral inactivation can be used for the preparation of monoclonal antibodies without adverse effects. This finding is especially important for monoclonal antibodies intended for therapeutic use although it is also of value for monoclonals used for other purposes (e.g., purification, diagnostics, etc.).

One of the advantages of monoclonal antibodies (Mabs) over plasma-derived antibodies (PD Abs) is that Mabs are expected to be free of viruses associated with plasma, especially human plasma viruses such as hepatitis viruses and human immunodeficiency virus (HIV). However, certain viruses are associated with cell lines from which the Mabs are derived.

For example, EBV is Epstein-Barr virus which is a common viral vector utilized for preparation of human monoclonals. See, for example, U.S. Pat. No. 4,446,465 to Lostrom. The concern here is that EBV is a human pathogen. We have found that exposure at pH 4.0 inactivates it very well without affecting the monoclonal antibody. Visna is a retrovirus and models human retroviruses reasonably well. Since tumorigenic potential for the hybridoma cell lines is a major concern demon-

stration of Visna inactivation is important. Mouse C (xenotropic) and Rauscher (Ecotropic) are murine retroviruses and their presence in murine cell is not too uncommon.

To determine the effect of pH on viral inactivation, the above Mabs were spiked with EBV, Visna and VSV with the results shown below.

#### EXAMPLES

##### *Pseudomonas aeruginosa* monoclonals

Mabs of the type described in U.S. Pat. No. 4,834,965 to Siadak et. al were incubated at a pH of 4.0 for at least 16 hours at 5° C. as an initial step in their overall purification.

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TABLE 4

Summary of Viral Inactivation/Clearance During PsMab IgM Purification	
Purification Step	Virus Reduction (Logs)
	EBV
Incubation pH 4	6.4

##### Factor VIII Monoclonals

Mabs identified as C7F7 and specific to human coagulation factor VIII were spiked with Visna virus and also subjected to a viral inactivation at pH 4.0 for various times shown below with the following results shown below.

TABLE 5

Clearance/Inactivation of Visna Virus During C7F7 Monoclonal IgG Purification	
IV. Virus Inactivation (pH 4.0) at 5° C.	
A. DEAE Eluate + Virus	
TIME 0 (pre-treatment)	6.0**
16 hours	2.25
24 hours	1.5
42 hours	1.5
48 hours	1.5

Note:  
Virus added to fresh aliquot of indicated preparation.  
\*Log TCID  
\*\*Log TCID/mL  
T.C. = Tissue culture

##### Tumor necrosis factor (TNF) Mabs

Mabs specific to TNF were spiked with the indicated viruses and reduction in titers as noted after exposure to pH 4.0 for 16 hours at 5° C. as shown below.

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TABLE 6

Model murine virus clearance during the TNF Mab purification process			
Tier Reduction of Indicated Virus (Log)			
Process step	Xenotropic Virus*		Ecotropic virus**
	Assay 1	Assay 2	Assay 2
pH 4.0 exposure for $\geq 16$	3.9	$\geq 3.6$	$\geq 12.3$

\*Log PFU, based on mink lung (S+L-) assay

\*\*Log PFU, based on XC plaque assay

TNF Mag is isolated from fermenter harvests of A10G10 hybridoma cells and processed through a multistep purification process which includes cell separation, polyethylene glycol precipitation, anion exchange chromatography, size exclusion chromatography and hydroxylapatite chromatography. Nonetheless, the level of trace impurities in the purified product needs to be estimated. These include cell substrate DNA, non-IgG murine proteins and media components. Concern for murine retroviruses in hybridoma cell lines has also necessitated the estimation of the clearance of relevant murine model viruses through the various steps of the purification process.

Model viruses representing various axonomic groups have been utilized to evaluate the ability of the purification process to fractionate and/or inactivate them. The overall clearance of relevant murine retroviruses included a low pH retrovirus inactivation step with virus titer reduction of  $\geq 10^{3.6}-10^{4.1}$ .

pH 4.0 was critical in this respect because further lowering of pH, although effective in retrovirus inactivation, results in loss of functional activity of the Mab.

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## CONCLUSION

This application is especially directed to inactivation of both known and unknown viruses associated with continuous (immortal) cells lines. These cell lines are known to those skilled in the art and includes hybridomas, transformed cells, genetically engineered cells, etc.

From the above example, it can be seen that exposure of various Mabs to a pH of about 4 for at least about 16 hrs at a temperature of at least about 5° C. is effective in inactivating viruses (decrease in viral titer of at least  $10^{3.6}$  fold). It is proposed that this approach would be effective in inactivating retroviruses and yet unknown viruses, particularly unknown retroviruses.

However, given the above disclosure, it is thought that variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention disclosed should be limited only by the following claims.

We claim:

1. A method of inactivating viruses in a solution of one or more monoclonal antibodies comprising the step of subjecting the solution to a pH of about 4.0 at a temperature of at least about 5° C. for at least about 16 hours to inactivate the viruses.

2. The method of claim 1 wherein in the antibodies are selected from the group consisting of anti-*Pseudomonas aeruginosa* monoclonal antibodies, anti-factor VIII monoclonal antibodies and anti-TNF monoclonal antibodies.

3. The method of claim 1 wherein the virus inactivated is selected from EBV, Visna, xenotropic and murine ecotropic viruses.

4. The method of claim 1 wherein the virus is a retrovirus.

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**United States Patent** [19]

Curry et al.

[11] Patent Number: 4,719,290

[45] Date of Patent: Jan. 12, 1988

[54] COMPOSITION OF INTRAVENOUS  
IMMUNE GLOBULIN[75] Inventors: Willie M. Curry, New Rochelle;  
David L. Farb, LaGrangeville, both  
of N.Y.[73] Assignee: Armour Pharmaceutical Corporation,  
Fort Washington, Pa.

[21] Appl. No.: 614,005

[22] Filed: May 25, 1984

## Related U.S. Application Data

[60] Division of Ser. No. 529,079, Sep. 2, 1983, Pat. No.  
4,482,483, which is a continuation-in-part of Ser. No.  
482,699, Apr. 6, 1983, abandoned.[51] Int. Cl.<sup>4</sup> C07G 7/00; A61K 35/10;  
A61K 39/00[52] U.S. Cl. 530/387; 530/363;  
424/85; 424/87; 424/101; 514/2; 514/6[58] Field of Search 530/350, 351, 387, 363;  
424/85, 87, 101; 514/2, 6

## [36] References Cited

## U.S. PATENT DOCUMENTS

2,520,076 8/1950 Williams et al. 530/387  
3,903,262 9/1975 Pappenhagen 530/387  
4,021,340 5/1977 Pollack et al. 424/864,124,576 11/1978 Coval 530/387  
4,126,605 11/1978 Schneider et al. 530/389 X  
4,168,303 9/1979 Nishida et al. 530/387  
4,379,086 4/1983 Kimura et al. 424/101 X  
4,396,608 8/1983 Tenold 424/101 X  
4,515,776 5/1985 Taniguchi et al. 530/387

## OTHER PUBLICATIONS

Smyth, Jr. et al., *Industrial Hygiene and Toxicology* 2nd  
Ed. "The Toxicology of the Polyethylene Glycols", pp.  
7-12, 1950.Miller et al., *J. Med. Microbiol.*, vol. 10, pp. 19-27, 1977.

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## [57] ABSTRACT

An intravenous immune globulin preparation having at least 99% pure globulin protein and an anticomplement activity of less than 0.10 C<sub>50</sub> units/mg IgG prepared by: precipitating impurities from Cohn Fraction II in an aqueous-alcohol medium at defined temperature and pH, removing the precipitated impurities, stabilizing the diluted solution with albumin, concentrating the solution and removing the alcohol therefrom. Also prepared by this method, an intravenous, hyperimmune globulin preparation having increased antibody titers to sixteen serospecific strains of *Pseudomonas aeruginosa*.

12 Claims, No Drawings

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## COMPOSITION OF INTRAVENOUS IMMUNE GLOBULIN

## RELATED APPLICATIONS

This application is a divisional of our copending application Ser. No. 529,079 filed Sept. 2, 1983, now U.S. Pat. No. 4,482,483, which in turn is a continuation-in-part of application Ser. No. 482,699 filed Apr. 6, 1983, now abandoned.

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention relates to high purity intravenously injectable gamma globulin (IgG) preparations and a process for preparing the same. More particularly, the invention relates to a product and method of making unmodified, unaltered, undenatured or native gamma globulin molecules of high purity for intravenous administration.

The invention also relates to high purity intravenous hyperimmune globulin preparations having increased antibody titers to sixteen serospecific strains of *Pseudomonas aeruginosa* and the method of making such preparations.

It has been known for some time that certain patients with humoral immunodeficiencies are susceptible to acute and chronic infections which sometimes assume life-endangering dimensions. These patients are not able to produce the required levels of antibodies and the same must be supplied to them for the prevention and treatment of such infections.

The immune globulin fraction of pooled human plasma contains antibodies to many viruses and bacteria and thus is effective in the management of various diseases including those associated with Staphylococci, Streptococci, Coli, *Pseudomonas*, *Herpes zoster* and *pyocyanus septicemia*.

Patients with normal levels of antibodies also require additional defense in overcoming serious infections such as caused by *Pseudomonas aeruginosa*.

Immunogenic *pseudomonas* vaccines and globulins having raised titers of protective antibody and increased phagocytic activity were found effective in the treatment of infections caused by *P. aeruginosa*.

Human immune globulins were first isolated on a large scale during the 1940's by F. J. Cohn. It was also observed that the aggregate formed during the fractionation procedure results in anticomplement activity and that clinical application causes adverse reactivity in the patient.

## 2. Description of the Prior Art

It has been known to prepare immune globulin containing antibodies by fractionating human blood plasma according to the so-called Cohn-method. It has also been known to further purify immune globulin for administration either intramuscularly or intravenously. While producing some of the desired effects, both kinds of administration have some disadvantages, which at times, may be serious or even life-threatening.

Intramuscular injections of immune globulin have proven effective in raising the level of circulating immune globulin and in decreasing the length, frequency and severity of infections in some patients. There are patients, however, who cannot achieve adequate immune globulin levels and protection from infection with intramuscular administration of immune globulin. Such patients when treated via plasma therapy experience

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improvement which indicates that intravenous administration may have advantages over the intramuscular route. Other disadvantages of intramuscular administration of immune globulin include the delayed onset of reaction resulting from the slow diffusion of the substance into the circulation, and inconsistent absorption and local degradation in the muscle where the injection is administered.

With intravenously administered immune globulin adequate levels of circulating antibody could be reached immediately and controlled by the rate of infusion. The intravenous route of administration also overcomes the effects of inconsistent absorption and local degradation in the muscle. Also, patients with small muscle mass or bleeding tendency tolerate an intravenous injection better than an intramuscular injection.

While intravenous administration is the preferred route of administration, the product so administered is not without some serious drawbacks. It is known that intravenously administered immune globulin may cause unpleasant side effects such as flushing, wheezing back and muscle pain, anxiety and hypotension. It has been observed that these side effects may be due to the activation of complement, secondary to the formation of immune complexes, aggregates of immune globulin and denatured globulin formed during the storage thereof.

The prior art has made great efforts to prepare immune globulin which has lesser anticomplement activity, mainly by decomposing or removing the aggregated or denatured globulin. Such efforts included: enzymatical hydrolysis using pepsin, plasmin, papain, or bacterial proteases; chemical treatment by an acid, propiolactone or the like; conversion of the immunoglobulin into a chemical derivative such as by amidation, alkylation or S-sulfonation; and fractional precipitation of the immunoglobulin using polyethylene glycol or the like.

While these methods seemed to decrease the presence of aggregated or denatured globulin in the final products and consequently lowered the anticomplement activity, they were not without other shortcomings, such as low activity of the antibody, shortened half-life time of the immunoglobulin in the blood, and the presence of some denatured impurities which is believed to cause a decrease in efficacy of the immunoglobulin.

To overcome the above-mentioned disadvantages, the prior art has further proposed various preparative methods for intravenous immunoglobulin. Illustrative of these are the methods disclosed in the following patents:

U.S. Pat. No. 4,256,631 discloses a process for the preparation of immunoglobulin for intravenous administration comprising the purification of immunoglobulin by a combination of a fractional precipitation method in which one or more divalent or trivalent metal salts are added to an aqueous solution of the immunoglobulin and the supernatant is processed by affinity chromatography using as an adsorbant a complex of human IgG and a polyhydroxy polymeric compound. The resultant immunoglobulin is said to be extremely pure.

U.S. Pat. No. 4,305,870 pertains to a method for making intravenous plasma derivatives which includes the steps of mixing bentonite and an aqueous solution of plasma derivatives containing exogenous activity, the bentonite and the mixing time being sufficient to adsorb exogenous activity, and isolating the aqueous phase from the bentonite. Optionally, for the removal of residual exogenous activity, the bentonite-treated aqueous

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phase is further purified by ion-exchange chromatography. The so-obtained product is said to have an acceptable low content of externally deleterious or exogenous activity.

While the above-noted attempts by the prior art greatly enhanced the success of treatment of various infectious diseases by producing satisfactory immunoglobulin for such treatment, none to our knowledge has produced a natural, unmodified, unaltered and undenatured product which desirably should have the following characteristics: it should contain substantially pure immunoglobulin G (IgG) so that it is substantially free of naturally occurring IgA and IgM antibodies; it should contain all subclasses of IgG, namely IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub> in substantially the ratio as they occur in blood plasma; spontaneous complement activation should be very low or absent; it should be free of polymeric IgG; and it should have a very low level of trace constituents, such as enzymes which tend to degrade and destabilize IgG during storage.

The underlying reason for the production of such a natural product will be easily ascertained by those skilled in the art from the following brief explanation.

The native IgG molecule is known to have two types of biological activities, namely, the immune-specific activity and the non-immune-specific or "effector" activity. The immune-specific activity is characterized by binding properties for specific antigens whereby the IgG molecule acts as an antibody. The non-immune-specific or "effector" activity includes binding and activation of complement, opsonic activity, and the binding to specific cellular receptors for the Fc portion of the molecule. Any change in the native IgG molecule which alters, reduces or eliminates either of these two types of activities is referred to as denaturation whether said denaturation is the result of intentional or unintentional chemical or enzymatic modification. Commercial preparations of intramuscular IgG, which are processed without chemical modifications, contain aggregated forms of IgG that cause high levels of spontaneously fixed and activated complement and are examples of unintentional denaturation. Examples of intentionally denatured IgG molecules include IgG preparations that are modified with the use of chemicals and/or enzymes in an attempt to improve the safety of intravenous administration. Such intentional denaturation diminishes or even completely eliminates the effector functions of IgG and reduces the total beneficial biological potency of the IgG.

In addition to the desired characteristics described above, a *Pseudomonas* immune globulin preparation must possess preformed, specific anti-*Pseudomonas* antibodies. Host defense for *Pseudomonas* depends upon the presence of adequate numbers of functional phagocyte cells plus serum opsonic activity. Optimal phagocytosis of *Pseudomonas* occurs in the presence of type-specific *Pseudomonas* antibody. At least seventeen separate strains of *P. aeruginosa* have been identified by the World Health Organization, many of which show unusual resistance to treatment with antimicrobial drugs. Each strain is characterized by localized infections that may overwhelm the host tissue. Endotoxin, toxin A, elastase and protease are released to further weaken the host's defensive mechanism. Clinical cases in which normal immune defenses are compromised, such as burn, cancer and cystic fibrosis cases are particularly susceptible to infection by *P. aeruginosa*. A fast acting intravenous injection of hyperimmune, polyva-

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lent gamma globulin to enhance specific antibody activity can be of life-saving to these patients.

Anti-*Pseudomonas* immune globulin, immune whole blood and immune plasma are known in the prior art. Notwithstanding their beneficial properties, their drawbacks include having limited antibody titers, protection against only some of the recognized strains of *P. aeruginosa*, and the lack of high purity.

It is, accordingly, an object of the present invention to provide a native gamma globulin preparation suitable for intravenous injection.

It is another object of the present invention to eliminate undesired denatured properties of IgG not by the alteration of effector functions but by the selective elimination of molecular forms of IgG which are denatured and at the same time eliminate impurities in the form of non-IgG proteins.

It is still another object of the present invention to provide a gamma globulin preparation suitable for intravenous administration, in which anticomplement activity is less than about 0.1 C<sub>50</sub> units/mg.

It is a further object of the present invention to provide a gamma globulin preparation containing at least 99.0% pure immune gamma globulin which is essentially free of IgA and IgM.

It is also an object of the present invention to provide a gamma globulin preparation containing all subclasses of IgG, namely IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub>, in substantially the ratio as occurring in normal blood plasma.

A further object of the present invention is to provide an intravenous, hyperimmune globulin preparation with increased antibody titers to sixteen serospecific strains of *Pseudomonas aeruginosa*.

A still further object of the present invention is to provide an intravenous, hyperimmune globulin preparation with increased antibody titers to sixteen serospecific strains of *Pseudomonas aeruginosa* in which anticomplement activity is less than about 0.1 C<sub>50</sub> units/mg.

Another object of the present invention is to provide an intravenous, hyperimmune globulin preparation which is essentially free of IgA and IgM.

It is still another object of the present invention to provide a simple economical process for commercial preparation of immune gamma globulin.

These and other objects and advantages of the present invention will be readily apparent to those skilled in the art from the description of the invention that follows.

#### SUMMARY OF THE INVENTION

According to the present invention, an unaltered, unmodified, undenatured or native immune gamma globulin preparation is provided for intravenous administration. Said preparation comprises at least 99.0% human native gamma globulin which has undergone no chemical or enzymatic modification, contains less than 0.1% IgA, essentially no IgM or aggregates and has an anticomplement activity of 0.1 or less C<sub>50</sub> units/mg. The immune gamma globulin preparation of the present invention consists of all the subtypes of IgG in approximately the same ratio as present in the starting material namely, about 64% IgG<sub>1</sub>, 29% IgG<sub>2</sub>, 6% IgG<sub>3</sub> and 1% IgG<sub>4</sub>.

The anti-*Pseudomonas* hyperimmune globulin embodiment of the present invention, in addition to having the above-described characteristics of immune gamma globulin, also possesses increased anti-body titers

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against sixteen serospecific strains of *Pseudomonas aeruginosa*. Hyperimmune globulin products herein referred to denote products having a greater quantity of antibodies than the quantity found in blood products obtained from un-immunized donors.

The process for the preparation of IgG includes the steps of:

- a., precipitation impurities from Cohn Fraction II or plasma fraction, harvested by plasmaphoresis in an aqueous-alcohol medium at a temperature of about 1°-10° C. and at a pH of about 7 to 9;
- b., removing the precipitated impurities;
- c., stabilizing the solution with albumin; and
- d., concentrating the solution and removing the alcohol therefrom.

The concentrated solution is formulated by the addition of a salt and/or with carbohydrates. The formulated IgG is sterile filtered and dispensed in vials.

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, the process for preparing the immune gamma globulin comprises the steps of:

- a., suspending proteins present in Cohn Fraction II obtained from normal or hyperimmune plasma in an aqueous solution of about 0 to 16% w/v and preferably about 2 to 10% w/v alcohol at a protein concentration of about 1 to 8% w/v and preferably about 1 to 4% w/v at a temperature of 1°-19° C. and preferably 2°-5° C;
- b., adjusting the pH of the suspension to about 7.8±0.4 and preferably to about 7.6±0.2;
- c., adding NaCl to the suspension to obtain a salt concentration of 0 to 2 mM NaCl;
- d., allowing the suspension to stand for 2 to 24 hours and preferably 6 to 18 hours to precipitate IgM, IgA, enzymes and polymeric forms of IgG impurities and to obtain equilibrium between precipitated impurities and dissolved IgG;
- e., removing the precipitated impurities by filtration or centrifugation to obtain a dilute IgG solution;
- f., stabilizing the dilute IgG solution by adding purified Human Serum Albumin to obtain an IgG/albumin ratio of 1/1 to 5/1 and preferably of 1/1 to 2/1;
- g., adjusting NaCl concentration of the solution to 0 to 0.9% w/v NaCl;
- h., adjusting the pH of the solution to about 6.9±0.4;
- i., concentrating the solution by ultrafiltration to obtain a concentration of 3.8 to 4.5% w/v IgG and preferably to about 4% w/v IgG;
- j., removing the alcohol and concentrating the solution to about 4 to 6% w/v IgG by dialfiltration; and
- k., formulating the solution with the addition of sodium chloride and/or with a carbohydrate.

According to the present invention, it has been found that impurities from native IgG can be separated without the use of chemicals that tend to denature IgG under precisely established conditions which are not suggested by the prior art and not predictable from the known behavior of the compounds used in the process. The conditions essentially consist of using low ionic strength alcohol solution, and cryoprecipitation at specific temperatures and pH.

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#### Starting Materials for the Preparation of Anti-Pseudomonas Hyperimmune Globulin

The polyvalent anti-Pseudomonas hyperimmune globulin of the present invention is produced by first isolating and combining immunizing antigens obtained from particular strains of *Pseudomonas aeruginosa* for use in a polyvalent vaccine which is capable of immunizing against any of sixteen recognized serotypes of *Pseudomonas aeruginosa* infection. Human volunteers then are vaccinated with the polyvalent vaccine to elicit responses in the antibody titers to all sixteen serotypes, followed by obtaining plasma from vaccinated donors and purification of the resultant high titer gamma globulin.

The prior art has used alternative serotyping schemes for *P. aeruginosa*, however, these are now being superseded by an internationally-approved serotyping system based, in the main, on Habs' original work (Habs, *J. Zschr. f. Hyg.* 144: 218-228, 1957). The sixteen strains used in the present invention have been serotyped by the Central Public Health Laboratory, Colindale, London and are stored in lyophilized form under security in the Wellcome Bacterial Culture Collection, London. Table I shows the sixteen serotypes under Wellcome Bacterial Collection and Designation Number and correspondence with the Habs serotypes.

TABLE I

Strains of <i>P. aeruginosa</i> Used for Production of Pseudomonas Vaccine		
Serotype	Wellcome Bacterial Collection No. (CN)	Wellcome Designation No.
HABS 1	6669	1
HABS 2	6670	2
HABS 3	6766	3
HABS 4	6767	4
HABS 5	6674	5
HABS 6	6675	6
HABS 7	6768	7
HABS 8	6677	8
HABS 9	6777	9
HABS 10	6789	10
HABS 11	6782	11
HABS 12	6709	12
VERNON 13	6710	13
MEITERT 10	6821	14
HOMMA 11	6787	15
HOMMA 13	6788	16

The procedure of isolating the antigens, preparing the polyvalent vaccine, immunizing volunteers and obtaining plasma therefrom is based on known techniques utilized by the prior art.

#### Vaccine

The vaccine may be prepared according to a publication by Miller et al, *J. Med. Microbiol.* Vol. 10, pp. 19-27, 1977.

Sixteen of the recognized virulent serotypes of *Pseudomonas aeruginosa* were identified in separate clinical isolates and cultured as sixteen separate master serotype broths. Each master culture was grown in the presence of ammonium lactate and other nutrients. (Aliquots of each master culture were frozen as starting materials for subsequent vaccine preparation.) The microorganisms were harvested by centrifugation, washed by resuspension and centrifugation in fresh media, then resuspended in an extraction solution containing glycine and ethylenediamine tetraacetic acid. The microorganisms were removed by centrifugation and destroyed. The extrac-

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tion solution contains cell wall components from the bacteria that are serotypically characteristic of each strain. The fresh extracts were treated with formalin to minimize toxicity. The ethylenediamine tetracetic acid and excess formalin were removed by dialysis and the extracts are concentrated until one ml of solution represents extract from  $10^8$  bacteria. Mice were immunized with each of the sixteen monovalent vaccines to elicit antibody responses corresponding to the bacterial strain, showing serospecificity in each case. The results were determined by the Enzyme-Linked Immunosorbent Assay (ELISA).

#### ELISA assay

The Elisa assay employing goat anti-human immunoglobulin G(IgG) conjugated with alkaline phosphatase (Sigma Chemical Co.) and p-nitrophenyl phosphate (Sigma Chemical Co.) as a indicator system is used for measurement of antibody response to the 16 individual pseudomonas serotypes contained in The Wellcome Pseudomonas Vaccine.

In brief, wells of Immulon I polystyrene microtiter plates (Dynatech Labs) are coated with 50  $\mu$ l of the individual serotype antigens in 0.1M glycine buffer (pH 9.5) by incubating at 4° C. for four hours. Optimal concentration of each serotype antigen is determined by block titration against Wellcome polyvalent pseudomonas antibody control. At the end of the incubation period, the plates are washed four times with 0.02M PBS-Tween 20 (pH 7.2).

Sera samples starting with a 1:50 dilution in PBS-Tween is diluted in twofold steps in 50  $\mu$ l amounts using an antidilutor (Dynatech Labs). The plates are then incubated at room temperature for three hours. Each plate contains appropriate positive and negative controls. After incubation, the plates are washed three times with PBS-Tween.

A predetermined optimal dilution of anti-human IgG conjugated with alkaline phosphatase in PBS-Tween is made and 50  $\mu$ l is dispensed into each well of the microtiter plates. The plates are then incubated at room temperature for one hour. After incubation, the plates are washed four times in PBS-Tween and 50  $\mu$ l of 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine (pH 9.8) is added. The plates are then incubated at 4° C. for 18 hours.

After incubation, 50  $\mu$ l of quenching solution 0.1M EDTA (pH 7.0) is added to each well. Optical density is determined spectrophotometrically for each well solution using a recording automatic spectrophotometer (Dynatech).

The antibody titer is determined by the maximal dilution of sample that gives an optical density of 0.3 or greater.

Generally, a correlation was found between a monovalent vaccine and consequent response thereto by inoculated mice, i.e. each serotype monovalent vaccine elicits an immune response to the particular serotype. A study by R. J. Jones and E. A. Roe (Br. J. Exp. Path. 56: 34-43, 1975) shows similar findings using haemagglutination assay.

#### Immunization

For human vaccination each of the sixteen nonovalent vaccines was blended and concentrated such that 1 ml of solution represents extract from  $10^8$  bacteria of each strain. One ml of the polyvalent vaccine was in-

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jected s.c. in volunteers three times at one week intervals to elicit antibody production.

An increase in plasma titer of antibodies for each of the sixteen serotypes occurred. Table II shows, however, that some individuals respond with a high average titer while others response with much lower titers as determined by the ELISA assay. The response as expressed by titer is usually maintained at a high level between three to six weeks post vaccination.

TABLE II

Days Following Immunization	Average of 16 Serotype Responses Relative to each Individual Zero Day Titer	
	High Response Group (15 Volunteers)	Low Response Group (13 Volunteers)
0	1.0	1.0
21	12.1	3.7
42	6.5	2.2
70	3.4	1.6
98	2.9	1.4
126	2.1	1.3

Accordingly, the time of collection of blood from immunized donors is of an important factor in obtaining immune globulin products having a high antibody level. Generally, about 3 weeks post immunization the antibody level appears to be the highest and decreases gradually thereafter.

#### Plasma Selection

The antibodies generated by human volunteers as a result of vaccination are harvested by plasmapheresis. Table III shows titer values on plasma pools obtained from 28 immunized donors, one group of which had high response and the other low response to immunization. It is also noted that plasma isolated from non-immunized donors also contains low titers of antibody to each of the sixteen serotypes of *P. aeruginosa*.

TABLE III

Serotype	RELATIVE RESPONSES IN THE TITER FOR EACH SEROTYPE OF PSEUDOMONAS AERUGINOSA IN SELECTED PLASMA POOLS			
	High Response Group		Low Response Group	
	Pre-Vaccination	21 Day Plasma Pool	Pre-Vaccination	21 Day Plasma Group
1	200	800	200	800
2	400	800	200	800
3	400	800	200	400
4	200	800	100	400
5	400	1600	200	800
6	100	800	50	200
7	200	800	50	400
8	200	800	100	400
9	200	800	200	400
10	200	1600	50	400
11	400	800	200	800
12	200	1600	200	400
13	25	800	25	400
14	400	1600	400	400
15	100	800	100	400
16	400	1600	200	400

#### Purification of Polyvalent Gamma Globulin

While both plasma pools, one having low and the other high antibody titer levels, may be used to produce immune gamma globulin, high titer plasma is preferred for use as starting material for the preparation of hyperimmune gamma globulin.

Before purification by the process of the present invention, coagulation factors, Factor VIII and Factor IX

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Complex are removed and the plasma subjected to Cohn fractionation. The purification procedure starts with the plasma fraction which is substantially equivalent to the Cohn Fraction II material.

#### Starting Materials for the Preparation of Immune Globulin (Normal Immune Globulin)

The starting material for normal immune gamma globulin may be the well-known Cohn Fraction II paste, or other starting materials having native IgG and its subclasses present. However, it is preferred to use Cohn Fraction II which has a history of safety and efficacy as a therapeutic product in the intramuscular dosage form and is commercially available. Alternatively, the active ingredient may be obtained by processing human plasma from which the coagulation factors, Factor VIII, and Factor IX Complex, are removed before the plasma is subjected to Cohn fractionation. Following the removal of Factor VIII and Factor IX, the Cohn cold-ethanol fractionation produces a series of protein fractions: Fraction I, Fraction II+III, Fraction IV<sub>1</sub>+IV<sub>2</sub>, and Fraction V. In addition to IgG, IgM and IgA, Fraction II+III is enriched in lipids, lipoproteins, pigmented materials such as carotinoids, as well as proteolytic enzymes. All of these substances must be separated from the IgG. After the separation of these undesirable substances, Fraction II is about 95% pure IgG and it represents about 40 to 50% of the IgG content of the starting plasma. Fraction II is comprised of all four subclasses of IgG, namely, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> and IgG<sub>4</sub>, in a similar ratio as that found in plasma. The 95% pure IgG is not suitable for direct formulation into an intravenous product for several reasons. IgG is easily denatured and as such, can form large high molecular weight aggregates. These aggregates can fix complement in the absence of antigen triggering a complement cascade which can be a risk to patients receiving intravenous infusions of IgG. The impurities amounting to about 5% in Cohn Fraction II are also undesirable, such as: IgM, which is easily denatured and readily fixes complement; IgA, which is known to cause anaphylactoid reactions in IgA-deficient patients; Pre-Kallikrein Activator which causes vasoactive effects on administration; Plasminogen/Plasmin, which can fragment IgG and lead to reduced circulatory half-life. Cohn Fraction II, therefore, is further purified and the native character of the molecule is preserved by careful handling and stabilization as further described in detail in the method of the present invention.

#### Process of Preparing IgG

Cohn Fraction II paste or its equivalent is suspended and soluble portions thereof is dissolved in an aqueous alcohol solution. While the preferred alcohol is ethanol, other pharmaceutically acceptable alcohols may also be used. Subsequent to precipitating the impurities consisting of IgM, IgA, enzymes, polymeric forms of IgG and other trace contaminants, the same are removed by either filtration or centrifugation. The filtration may be accomplished by adding a diatomaceous earth filter aid, such as Hyflo-Super Cel, to the suspension, mixing the same with the suspension and filtering through 0.2 to 0.5 micron filter pads, such as Cuno 60SP. Alternatively, the precipitate may be removed by centrifugation using conventional equipment. The filtrate is concentrated by ultrafiltration over semipermeable membranes such as Pellicon system containing PT series membranes with a 10,000 or 20,000 m. w. limit in conjunction with a 1.5

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micron filter, such as a Pall filter cartridge. The pH is adjusted to  $7.3 \pm 0.5$ , albumin is added to stabilize the purified gamma globulin and NaCl is added to improve the solubility. The alcohol is removed from the IgG solution by diafiltration at constant volume using 5 to 10 volumes of 0.2% w/v NaCl. After the removal of the alcohol the solution is concentrated further to about 6.0% w/v IgG. The alcohol-free IgG/albumin solution is adjusted to pH  $6.9 \pm 0.4$  using citric acid. This solution then is diluted to contain about 5% w/v IgG by formulating the same with a salt or sugar solution. If formulated with salt, the final concentration of salt should be about 0.9% w/v. If formulated with sugar, the sugar concentration, in general, should be in the range of about 2.5 to 10% w/v. Such concentrations will vary according to the particular sugar used, for example, for monosaccharides the final concentration should be about 5.0% w/v, for disaccharides about 10.0% w/v. In addition, formulations may be made with sugar/salt combinations, such as 2.5% w/v glucose with 0.45% w/v NaCl, or 5.0% w/v sucrose with 0.45% w/v NaCl. The thus formulated IgG solution is filtered through 0.2-0.5 micron pads or cartridges, such as Cuno 60SP or Cuno IDEP, followed by filtration through a series of clarification and sterilization filters having a porosity of from 1.5 to 0.2 microns.

It is to be noted that the process of the present invention may be used to prepare a variety of hyperimmune globulin products containing hyperimmune globulin against one or more serotypes of *Pseudomonas aeruginosa*. In addition, the purification process may also be used for obtaining highly purified hyperimmune globulin that is effective against other pathogens. Still further, it is also contemplated that polyvalent immune globulin products for intravenous use having increased titers against at least two, but preferably against all sixteen, serotypes of *Pseudomonas aeruginosa* produced by the method of the present invention or by other appropriate art recognized methods are within the purview of the present invention.

The following examples further illustrate the invention:

#### EXAMPLE 1

1 Kg Cohn Fraction II paste was suspended in 10 liters of cold purified water. The pH was adjusted to 7.5 and the suspension was allowed to stand for 4 hrs. at  $2 \pm 1.0^\circ \text{C}$ . The precipitate which contains aggregated IgG, IgM, PKa, plasminogen and other trace contaminants was removed by centrifugation at  $3000 \times g$  for 20 min. or by filtration through a 0.2-0.5 micron filter pads such as Cuno 60 SP. The solution was adjusted to pH 6.8, stabilized by adding albumin at a ratio of 2 parts IgG to 1 part albumin, concentrated, diafiltered using 0.2% w/v NaCl and formulated by adding the desired sugar and/or salt. The solution was readjusted to pH 6.8 and diluted to a solution that had a final concentration of 5.0% w/v IgG with the desired concentration of salt and/or sugar.

#### EXAMPLE 2

1 Kg Cohn Fraction II was suspended in 15 liters of a cold purified water ethanol solution so that the final alcohol concentration was 4% w/v. The pH was adjusted to 7.6 and the suspension was allowed to stand for 2 hrs. at  $2 \pm 1.0^\circ \text{C}$ . The precipitate was removed, albumin was added at a ratio of 2 parts IgG to 1 part albumin, concentrated, diafiltered at constant volume

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using 0.2% w/v NaCl, and formulated by adding the desired sugar and/or salt. The pH was adjusted to 6.8 and the solution is diluted to a 5% w/v solution which contained the desired concentration of salt and/or sugar.

## EXAMPLE 3

1 Kg Cohn Fraction II was suspended in 15 liters of cold water and alcohol was added to the suspension to obtain a final concentration of 4% w/v alcohol. The suspension was adjusted to pH 7.6 and allowed to stand for 2 hrs. at  $2 \pm 1.0^\circ \text{C}$ . The precipitate was removed and the filtrate was adjusted to pH 6.8. Albumin is added at a ratio of 1 to 2. The solution was concentrated, diafiltered at constant volume using cold Pyrogen Free water. The solution was diluted to a 5% w/v IgG solution which contained the desired concentration of salt and/or sugar. The pH of the solution was readjusted to pH 6.8, passed through clarification and sterilization filters, aseptically filled into sterile bottles, stoppered and sealed.

## EXAMPLE 4

1 Kg Cohn Fraction II was suspended in 20 liters of a cold purified water-ethanol solution so that the final alcohol concentration was 4% w/v. The pH was adjusted to pH 7.4 and the suspension is allowed to stand 16 hrs. at  $2 \pm 1.0^\circ \text{C}$ . The precipitate was removed and the filtrate was adjusted to pH 6.8. Albumin was added at a ratio of 1—1 and the solution was concentrated and diafiltered at constant volume using 10 volumes of cold Pyrogen Free water at  $2 \pm 1.0^\circ \text{C}$ . The solution was then formulated by adding the desired sugar and/or salt. The pH was readjusted to 6.8 and the solution was diluted to contained a 5% w/v IgG solution that contains the desired concentration of salt and/or sugar.

## EXAMPLE 5

Native IgG was isolated from a pool of plasma obtained from 15 non-immunized donors by Cohn fractionation and purification according to the process of the present invention. Antibody titers of the pooled plasma and the purified gamma globulin were measured. Side-by-side results of titers for plasma and IgG are shown for all 16 serotypes of *Ps. aeruginosa* in Table IV.

TABLE IV

Antibody Titers of Plasma and Pure IgG of Non-Immunized Donors		
Serotype	Plasma Pool Titer (approx. 1% IgG)	Titer/1% Purified IgG
1	100	200
2	400	200
3	400	200
4	100	100
5	400	400
6	25	200
7	100	100
8	100	100
9	200	100
10	200	200
11	200	200
12	50	100
13	25	100
14	400	400
15	100	200
16	200	200

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## EXAMPLE 6

Native IgG was isolated from a large commercial pool (about 2,000 donors) of normal, non-immunized donors and processed as in Example 5. Antibody titers of the purified gamma globulin were measured. Results are shown for all 16 serotypes of *Ps. aeruginosa* Table V.

TABLE V

Serotype	Titer/1% Purified IgG
1	400
2	400
3	400
4	200
5	800
6	200
7	200
8	200
9	200
10	100
11	400
12	200
13	200
14	400
15	200
16	400

## EXAMPLE 7

Thirteen low-response donors that were immunized with polyvalent pseudomonas vaccine were selected for plasmapheresis at three weeks post vaccination. Native IgG was isolated from the plasma pool obtained from these donors as in Example 5. Antibody titers of the pooled plasma and the purified gamma globulin were measured. Side-by-side results of titers for plasma and IgG are shown for all 16 serotypes in Table VI.

TABLE VI

Low-response Donors (3 weeks post-vaccination)		
Serotype	Plasma Pool Titer	Titer/1% Purified IgG
1	800	400
2	800	800
3	400	800
4	400	400
5	800	400
6	200	400
7	400	400
8	400	400
9	400	400
10	400	400
11	800	800
12	400	800
13	400	200
14	400	800
15	400	400
16	400	800

## EXAMPLE 8

Thirteen high-response donors that were immunized with polyvalent pseudomonas vaccine were selected for plasmapheresis at three weeks post-vaccination. Native IgG was isolated from the plasma pool obtained from the donors as described in Example 5. Antibody titers of the pooled plasma and the purified gamma globulin were measured. Side-by-side results of titers for plasma and IgG are shown for all 16 serotypes in Table VII.

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TABLE VII

High-response Donors (3 weeks post-vaccination)		
Serotype	Plasma Pool Titer	Titer/1% Purified IgG
1	800	800
2	800	1600
3	800	800
4	800	800
5	1600	1600
6	800	800
7	800	800
8	800	1600
9	800	800
10	1600	1600
11	800	1600
12	1600	3200
13	800	800
14	1500	3200
15	800	1600
16	1600	1600

## EXAMPLE 9

Fifteen high-response donors that were immunized with polyvalent pseudomonas vaccine were selected for plasmapheresis at three weeks post-vaccination. Native IgG was isolated from the plasma pool obtained from the donors as described in Example 5. Antibody titers of the pooled plasma and the purified gamma globulin were measured. Side-by-side results of titers for plasma and IgG are shown for all 16 serotypes in Table VIII.

TABLE VIII

High-response Donors (3 weeks post-vaccination)		
Serotype	Plasma Pool Titer	Titer/1% Purified IgG
1	800	800
2	800	800
3	800	800
4	800	800
5	800	800
6	400	800
7	400	800
8	800	800
9	800	800
10	800	1600
11	800	800
12	800	1600
13	800	800
14	1600	1600

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TABLE VII-continued

High-response Donors (3 weeks post-vaccination)		
Serotype	Plasma Pool Titer	Titer/1% Purified IgG
15	800	1600
16	800	1600

## EXAMPLE 10

Fifteen high-response donors that were immunized with polyvalent pseudomonas vaccine were selected for plasmapheresis at eighteen weeks post-vaccination. Native IgG was isolated from the plasma pool obtained from these donors as described in Example 5. Antibody titers of the pooled plasma and the purified gamma globulin were measured. Side-by-side results of titers for plasma and IgG are shown for all 16 serotypes in Table IX.

TABLE IX

High-response Donors (18 weeks post-vaccination)		
Serotype	Plasma Pool Titer	Titer/1% Purified IgG
1	400	400
2	800	400
3	800	400
4	400	400
5	400	800
6	200	200
7	400	400
8	400	400
9	400	400
10	400	400
11	400	400
12	400	800
13	400	200
14	400	800
15	400	400
16	400	800

## EXAMPLE 11

IgG obtained in Examples 5, 6, 7, 9 and 10 were studied to determine their efficacy in the burned mouse model based on Stieritz & Holder (J. Infect. Dis. 131: 688-691, 1975). As apparent from Tables X and XI, antibody titers tend to correlate with survival of the mice infected with  $10^6$  *P. aeruginosa*.

TABLE X

Survival of Burned, Infected Mice Treated With Various Intravenous IgG Preparations (5 mg)				
Treatment	Examples	Infective Dose	% Survival 5 Days	Serotype 2/5 Titer
None		$10^6$ <i>P. aeruginosa</i> Serotype 2/5	0	50
Albumin		$10^6$ <i>P. aeruginosa</i> Serotype 2/5	0	50
Normal Gamma Globulin 15 Donors	5	$10^6$ <i>P. aeruginosa</i> Serotype 2/5	20	1350
Normal I.V. Gamma Globulin	6	$10^6$ <i>P. aeruginosa</i> Serotype 2/5	60	2300
3 Weeks Post Vaccination 13 Refractory Donors	7	$10^6$ <i>P. aeruginosa</i> Serotype 2/5	60	2300
3 Weeks Post Vaccination 15 Responsive Donors	9	$10^6$ <i>P. aeruginosa</i> Serotype 2/5	80	5600
18 Weeks Post Vaccination 15 Responsive Donors	10	$10^6$ <i>P. aeruginosa</i> Serotype 2/5	60	2300

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TABLE XI

Survival of Burned, Infected Mice Treated With Various Intravenous IgG Preparations (5 mg)				
Treatment	Examples	Infective Dose	% Survival 5 Days	Serotype 6 Titer
None		$10^6$ <i>Pt. aeruginosa</i>	0	50
		Serotype 6		
Albumin		$10^6$ <i>Pt. aeruginosa</i>	0	50
		Serotype 6		
Normal Gamma Globulin	5	$10^6$ <i>Pt. aeruginosa</i>	0	700
15 Donors		Serotype 6		
Normal I.V. Gamma	6	$10^6$ <i>Pt. aeruginosa</i>	0	700
Globulin		Serotype 6		
3 Weeks Post Vaccination	7	$10^6$ <i>Pt. aeruginosa</i>	40	900
13 Refractory Donors		Serotype 6		
3 Weeks Post Vaccination	9	$10^6$ <i>Pt. aeruginosa</i>	40	1800
15 RESPONSIVE DONORS		Serotype 6		
18 Weeks Post Vaccination	10	$10^6$ <i>Pt. aeruginosa</i>	60	3600
15 Responsive Donors		Serotype 6		

The products obtained by the foregoing examples, 20 and products made according to the teaching of the specification, in addition to having been tested for antibody titers and efficacy as previously described, were tested by using appropriate procedures for verifying and defining other characteristics of IgG products. In 25 general, the following qualities characterized the products of the present invention when analyzed according to the methods identified below.

A product of the present invention is at least 99% pure immune gamma globulin; it is essentially free of 30 IgA and IgM as measured by Radial Immune Diffusion (RID) according to Mancini, G., Caronara, A. D., Hermans Immuno-chemistry 2 235 (1975) and by the laser nephelometry method based on antibody-antigen complex measurements as described by Schultz et al., J. 35 Immunological Methods 31 31-40 (1979). No plasma or plasminogen were detected by the use of streptokinase and CBZ-lyz-P-nitrophenol, as described by the method by Silverstein, R. M. Analytical Biochemistry 65 500-506 (1975).

Anticomplement activity (ACA) is less than 0.10 C'50 units/mg IgG as measured by a modified method of Kabet, E. A. and Mayer, M. Experimental Immunoch- 40 emistry, Second Edition, Thomas Springfield (1961). Aggregated IgG was not detected when the product was assayed either by high performance liquid chro- 45 matography (HPLC) using a TSK 3000 SW column or by gel permeation chromatography using a 90 cm. column packed with LKB ultragel AcA 34. The product does not contain detectable hepatitis surface antigen B 50 when measured by the radioimmune assay using RIAS- URE II test kit obtained from Electro-Nucleonics Laboratories, Inc. The product contains all the subtypes of IgG, namely IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub>, and the per- 55 centage for each of these subtypes is about 64%, 29%, 6%, and 1% respectively, which is essentially identical to the percent distribution found in the Cohn Fraction II paste as measured by Radial Immune Diffusion (RID) method referred to above. The antibody titers for mea- 60 sles, polio, diphtheria and hepatitis were equivalent to the titers found in the commercial intramuscular im- mune serum globulin products.

As earlier indicated, the pure IgG molecule obtained according to the method of the present invention is formulated into pharmaceutical dosage forms suitable 65 for intravenous administration. Such dosage forms include the lyophilized form and the liquid dosage form of IgG.

In the lyophilized dosage form a pharmaceutically acceptable sugar such as maltose, sucrose or glucose is added to the pure product to protect the IgG and to provide bulk during freezing and lyophilization. An example for such lyophilized composition is given in Example 12, which was found to be stable for at least one year at both refrigeration and room temperatures with no change in anticomplement activity which averaged between 0.03 to 0.04 C'50 units/mg. No aggregates or fragments were detected.

## EXAMPLE 12

Ingredients	gms/50 ml
Immune Globulin	2.5
Normal Serum Albumin	1.25
Sodium Chloride	0.1
Maltose	5.0
Water for Injection*	q.s. to 50 ml

(\*water is removed by freeze-drying)

Examples 13, 14 and 15 show liquid dosage formula- tions containing 5% w/v IgG and 2.5% w/v Normal Serum Albumin with either 10% maltose, 5% sucrose, or no carbohydrate. Appropriate amounts of sodium chloride was added in each case to make the prepara- tions iso-osmotic.

Upon testing the maltose-containing IgG was found to be stable at room temperature for 6 months and at refrigeration temperatures for at least a year. The anti- complement activity did not change significantly from the initial levels and averaged between 0.025 and 0.045 C'50 units/mg. In addition, no aggregates or fragments were detected. The liquid formulations of IgG contain- ing sucrose or no carbohydrates were found to be stable for at least 6 months at both refrigeration and room temperatures. The anticomplement activity did not change significantly from the initial levels, and aver- aged between 0.07 and 0.1 C'50 units/mg. As with the previous formulation, no aggregate or fragments were 60 detected.

## EXAMPLE 13

Ingredients	gms/50 ml
Immune Globulin	2.5
Normal Serum Albumin	1.25
Sodium Chloride	0.1
Maltose	5.0

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-continued

Ingredients	gms/50 ml
Water for Injection	q.s. to 50 ml

## EXAMPLE 14

Ingredients	gms/50 ml
Immune Globulin	2.5
Normal Serum Albumin	1.25
Sodium Chloride	0.25
Sucrose	2.5
Water for Injection	q.s. to 50 ml

## EXAMPLE 15

Ingredients	gms/50 ml
Immune Globulin	2.5
Normal Serum Albumin	1.25
Sodium Chloride	0.45
Water for Injection	q.s. to 50 ml

Specific results on formulations prepared according to the present invention are shown in Table XII.

TABLE XII

Analysis of IV-IGG Lots						
Immunoglobulin Composition						
Lot No.	Description	% IgG	% IgA	% IgM	ACA C50 U/mg	Mol. Size % Aggregate
1	Lyophilized 10% Maltose	100	0	0	0.040	0
2	10% Maltose	99.87	0.03	0	0.049	0
3	5% Glucose	99.97	0.03	0	0.053	0
4	10% Maltose	100	0	0	0.045	0
5	10% Maltose	99.97	0.03	0	0.030	0
6	Saline	99.97	0.03	0	0.082	0
7	5% Sucrose	99.96	0.04	0	0.077	0
8	Saline	100	0	0	0.088	0
9	10% Maltose	99.95	0	0	0.033	0
10	10% Maltose	99.91	0.09	0	0.041	0
11	5% Maltose	99.96	0.04	0	0.087	0

Table XIII shows an analysis of IgG of the present invention versus that of commercial products.

TABLE XIII

ANALYSIS OF IgG of the PRESENT INVENTION VS COMMERCIAL IgG PRODUCTS									
Manufacturer	IgG Mg/dl	IgA mg/dl	IgM mg/dl	IgG <sub>1</sub> %	IgG <sub>2</sub> %	IgG <sub>3</sub> %	IgG <sub>4</sub> %	ACA C50/mg	Pka % of BOB Ref. 2*
Commercial Product 1	4660	5.7	0	52.9	41.5	4.01	1.18	0.046	4.8
Commercial Product 2	2520	35.3	9.3	78.1	11.1	6.98	3.36	0.062	5.3
Commercial Product 3	4687	0	5.5	55.6	38.0	1.5	4.71	0.087	58.4
Commercial Product 4	5070	28.4	36	72.86	25.76	0	1.38	0.015	31.0
Present Invention	3520	0	0	63.6	28.6	6.3	1.5	0.045	0.26

\*% of Pka (Pyr-Kallikrein Activator) obtained from the FDA office of Biologics.

The formulations of the present invention are administered intravenously. Generally, an amount of 1 to 10 gm of gamma globulin may be used at a time. However, the dose of gamma-globulin for use in intravenous administration depends on the age, physical condition, antibody titer of the particular formulation, etc. and as such the physician will determine the particular dose suitable for effecting treatment based on his considering the various factors and circumstances.

It is apparent that numerous modifications and variations of the invention may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and

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the invention is limited only by the scope of the appended claims.

What is claimed is:

1. A process for the preparation of an unmodified, native gamma immune globulin for intravenous administration comprising the steps of:
  - a., suspending of IgG Cohn Fraction II in an aqueous solution of about 2 to 10% w/v ethanol at a protein concentration of about 1 to 4% w/v at a temperature of 2°-5° C;
  - b., adjusting the pH of the suspension to about 7.6±0.2;
  - c., adding NaCl to the suspension to obtain salt concentration of 0 to 2 mM NaCl;
  - d., allowing the suspension to stand for 6 to 18 hrs. to precipitate IgM, IgA, enzymes, polymeric forms of IgG impurities and to obtain equilibrium between precipitated impurities and dissolved IgG;
  - e., removing the precipitated impurities to obtain a dilute IgG solution;
  - f., stabilizing the dilute IgG solution by adding Human Serum Albumin to obtain an IgG/albumin ratio of 1/1 to 2/1;
  - g., adjusting NaCl concentration of the solution to 0 to 0.9% w/v NaCl;
  - h., adjusting the pH of the solution to about 6.9±0.4;
  - i., concentrating the solution by ultrafiltration to obtain a concentration of about 4% w/v IgG;
  - j., removing the ethanol and concentrating the solution to about 4 to 6% w/v IgG by diafiltration; and
  - k., formulating the solution with the addition of sodium chloride, carbohydrates or combinations thereof.
2. The process of claim 1 wherein said Cohn Fraction II is obtained from pooled plasma of *Pseudomonas aeruginosa* immunized donors.
3. The process of claim 1 wherein said Cohn Fraction II is about 95% pure, native gamma globulin.

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4. The process of claim 1 wherein said Cohn Fraction II is about 95% pure, native hyperimmune globulin.

5. The process of claim 3 wherein said 95% pure native gamma globulin comprises IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub> and IgG<sub>4</sub>.

6. The process of claim 1 wherein the precipitated impurities are removed by filtration.

7. The process of claim 1 wherein the precipitated impurities are removed by centrifugation.

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8. The process of claim 1 wherein the IgG solution is formulated with NaCl to obtain a final concentration of about 0-0.9% w/v NaCl.

9. The process of claim 1 wherein the IgG solution has a carbohydrate concentration of about 2.5 to 10% w/v.

10. The process of claim 9 wherein said carbohydrate is glucose.

11. The process of claim 9 wherein said carbohydrate is sucrose.

12. The process of claim 1 wherein said unmodified, native, immune globulin is hyperimmune globulin.

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US005486293A

## United States Patent [19]

Boschetti et al.

[11] Patent Number: 5,486,293

[45] Date of Patent: Jan. 23, 1996

## [54] REMOVAL OF SMALL EXOGENOUS MOLECULES FROM BIOLOGICAL FLUIDS

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[73] Assignee: Hemosure, Inc., Marlborough, Mass.

[21] Appl. No.: 215,201

[22] Filed: Mar. 21, 1994

[51] Int. Cl.<sup>6</sup> B01D 15/00; B01D 15/08

[52] U.S. Cl. 210/635; 210/656; 530/413; 530/417

[58] Field of Search 210/635, 644, 210/645, 650, 656; 422/101; 436/177, 178; 530/413, 414, 416, 417

## [56] References Cited

## U.S. PATENT DOCUMENTS

4,481,189	11/1984	Prince	
4,540,573	9/1985	Neurath et al.	514/2
4,615,886	10/1986	Purcell et al.	514/2
4,720,385	1/1988	Lembach	424/86
4,764,369	8/1988	Neurath et al.	424/89
4,841,023	6/1989	Horowitz	530/351
4,857,514	8/1989	Lippa et al.	514/78
4,939,176	7/1990	Seng et al.	514/724
5,053,135	10/1991	Boschetti et al.	210/635
5,151,499	9/1992	Kameyama et al.	530/381
5,186,945	2/1993	Shanbrom	424/529
5,204,324	4/1993	Shanbrom	514/2
5,268,097	12/1993	Girot et al.	210/198

## FOREIGN PATENT DOCUMENTS

0239859 of 1987 European Pat. Off.

## OTHER PUBLICATIONS

Prince et al. "Inactivation of Hepatitis B and Hutchinson Strain Non-A, Non-B Hepatitis Viruses . . ." *Vox Sang* 46, 36-43 (1984).

Prince et al. "Quantitative Assays for Evaluation of

HTLV-III Inactivation . . ." *Cancer Research* 45, 4592s-4594s (1985).Prince et al. "Sterilisation of Hepatitis and HTLV-III Viruses by Exposure to . . ." *The Lancet*, Mar. 29, 1986, pp. 706-709.Horowitz et al. "Inactivation of viruses in labile blood derivatives" *Transfusion* 25, 516-522 (1985).Edwards et al. "Tri (n-Butyl) Phosphate/Detergent Treatment of Licensed Therapeutic . . ." *Vox Sang* 52, 53-59 (1987).Horowitz et al. "Inactivation of Lipid-Enveloped Viruses in Labile Blood Derivatives . . ." *Vox Sang* 54, 14-20 (1988).Horowitz et al. "Solvent/Detergent-Treated Plasma: A Virus-Inactivated Substitute . . ." *Blood* 79, 3, 826-831 (1992).DiPaolantonio, et al. "Low Risk of Transmission of the Human Immunodeficiency . . ." *J. Med. Virology* 36, 71-74 (1992).Piquet et al. "Virus Inactivation of Fresh Frozen Plasma by a Solvent Detergent Procedure: . . ." *Vox Sang* 63, 251-256 (1992).Michalski et al. "Large-Scale Production and Properties of a Solvent-Detergent-Treated . . ." *Vox Sang* 55, 202-210 (1988).Strancar et al. "Extraction of Triton X-100 and its determination in virus-inactivated . . ." *J. Chrom. A* 658, 475-481 (1994).L. Guerrier et al., *Journal of Chromatography B: Biomedical Applications*, 664, pp. 119-125 (1995).

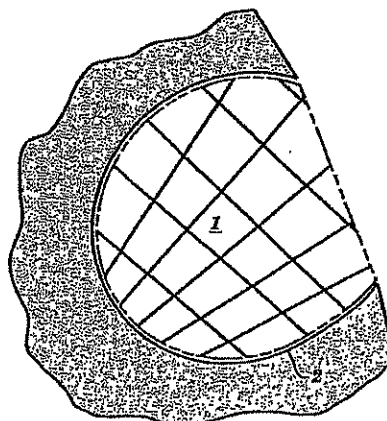
Primary Examiner—John Kim

Attorney, Agent, or Firm—Heslin &amp; Rothenberg

## [57] ABSTRACT

Novel sorbents and methods for removing small hydrophobic and amphophilic molecules from biological fluids were disclosed. The methods and materials were particularly useful for removing viral inactivating agents from blood and blood fractions. The novel sorbents comprise a porous mineral oxide matrix having its interior porous volume substantially filled with a crosslinked hydrophobic polymer network.

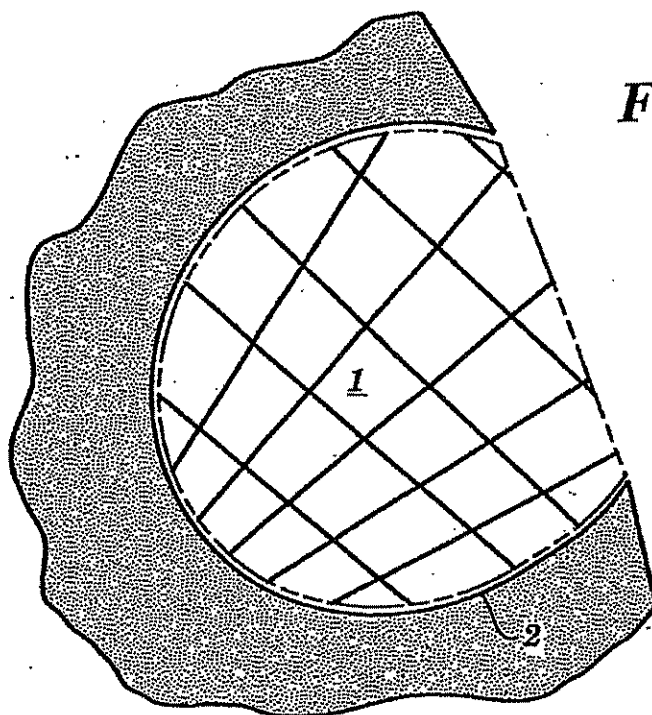
16 Claims, 1 Drawing Sheet



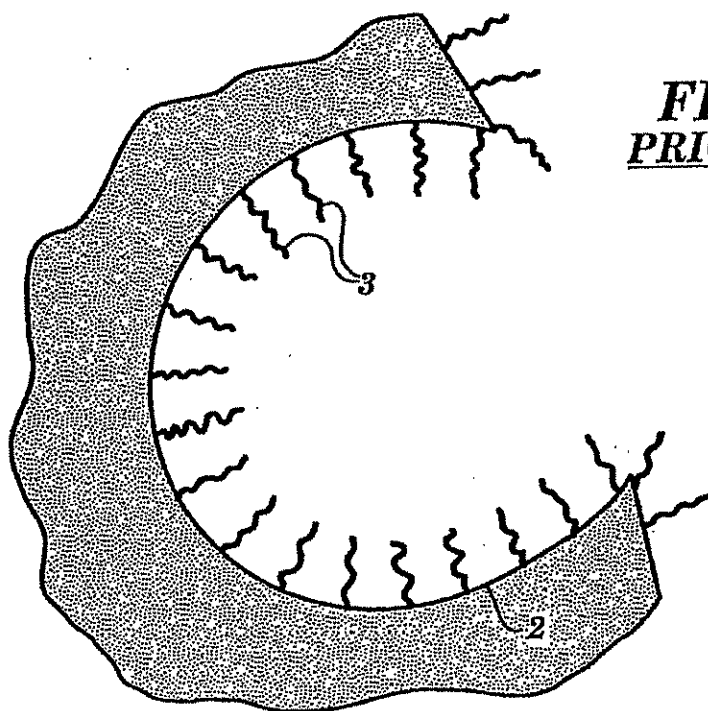
U.S. Patent

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**FIG. 1**



**FIG. 2**  
**PRIOR ART**

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## REMOVAL OF SMALL EXOGENOUS MOLECULES FROM BIOLOGICAL FLUIDS

### FIELD OF THE INVENTION

The invention relates to a method and materials for removing small exogenous molecules such as viral inactivating agents from biological fluids such as blood and blood fractions.

### BACKGROUND OF THE INVENTION

Numerous attempts have been made to inactivate viruses such as Hepatitis B (HB), non-A, non-B Hepatitis (NANBH), Human T Lymphotropic Retrovirus Type 3 (HTLV), Human Immunodeficiency Virus (HIV), and Lymphadenopathy Associated Virus (LAV). At present, the method of choice for inactivating these viruses in blood and blood fractions is treatment with a solvent such as tri-n-butyl phosphate and a detergent such as polysorbate 80 (Tween 80) or sodium cholate. Much of the early work in this area was done by the group of Bernard Horowitz and Alfred Prince at the New York Blood Center and as of February 1991, over 1.7 million doses of solvent and detergent treated coagulation factor concentrates had been infused.

In addition to tri-n-butyl phosphate, other phosphate esters, ether and halohydrocarbons have been described as useful solvents. In addition to polysorbate or sodium cholate detergents, other nonionic surfactants, particularly ethoxylated octylphenols and nonylphenols, as well as sulfobetaines, phosphatidyl cholines and octyl  $\beta$ -D-glucopyranoside have been mentioned as viral inactivating agents. For example, U.S. Pat. No. 4,540,573 describes the use of a number of organic solvent and detergent pairs to reduce the infectivity of hepatitis and other viruses.

In all of the foregoing treatments, exogenous agents are added to the biological fluid. In most cases, these exogenous agents must be removed from the biological fluid before it can be administered to a human. European application 239,859 describes a method that is currently employed to remove lipid soluble process chemicals from biological fluids. It comprises bringing the fluid into contact with a naturally occurring oil, agitating the resultant mixture, separating the phases by sedimentation or centrifugation, decanting the upper lipid phase, and utilizing the residual biological fluid. Aside from the mechanical complexity of this process, it appears applicable only to the removal of lipid soluble process chemicals (such as tri-n-butyl phosphate). Indeed the application indicates that a common non-ionic surfactant (polysorbate 80) is poorly extracted.

Gel filtration has also been proposed for removing detergents and solvents from blood fractions based on molecular weight differences. Horowitz et al. [*Transfusion*, 25, p. 516-522 (1985)] have described the removal of tri-n-butyl phosphate from anti-hemophilic factor concentrates by chromatography on Sephadex G-25; however, gel chromatography is not a practical method for removing solvent and detergent from whole blood. Moreover, it was ineffective for the removal of polysorbate 80 from a blood component, although effective for removing sodium cholate. Horowitz et al. [*Blood*, 79, p. 826-831 (1992)] have also suggested the removal of tri-n-butyl phosphate and Triton® X-100 (polyethoxylated octylphenol) from fresh frozen plasma by extraction with soybean oil, centrifugation, and then preparative chromatography on C-18 reverse phase.

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None of the methods presently in use or proposed is particularly attractive for the routine processing of blood and blood fractions. There is thus a need for a simple and effective method for removing small exogenous molecules, both hydrophobic and polar, from blood and other biological fluids.

It is therefore an object of the present invention to provide a method for removing small exogenous molecules from a biological fluid quickly and efficiently.

It is another object of the invention to provide a method that can remove exogenous molecules without impairing the function of the biological fluid.

It is a further object to provide a method for removing exogenous molecules that can remove both hydrophobic and amphiphilic molecules.

It is a more particular object of the present invention to provide a method for removing viral inactivating agents from blood or blood fractions quickly and efficiently in a clinical setting.

It is a further object of the invention to provide a porous support suitable for removing small exogenous molecules without impairing the function of the biological fluid.

These and other objects, features and advantages are provided by the instant invention summarized below.

### SUMMARY OF THE INVENTION

In one aspect, the invention relates to a method for removing contaminants from a biological fluid. The method comprises bringing the fluid into contact with a cross-linked hydrophobic polymer network which fills the pores of a mineral oxide matrix. The cross-linked polymer network overlays the porous mineral oxide and fills the interior porous volume but is not covalently bound thereto. Hydrophobic and amphiphilic molecules of molecular weight below 10,000 Daltons are simultaneously removed from the biological fluid as it passes over the mineral oxide supported hydrophobic polymer network.

In particular, the method may be used for removing solvents and surfactants from biological fluids. Preferred biological fluids include blood, blood fractions and biological extracts from which viral inactivating agents may be removed.

Preferred mineral oxide matrices have initial average particle sizes of about 5 to about 2,000 microns, porous volumes from about 0.2 to about 4 cubic centimeters per gram, surface areas from about 1 to about 1000 square meters per gram and initial pore sizes from about 50 to about 6,000 angstroms. Most preferably the mineral oxide matrix has an initial porous volume of about 1 cubic centimeter per gram and an initial surface area of about 200 square meters per gram.

The cross-linked hydrophobic polymer may be selected from the group consisting of acrylates, methacrylates, acrylamides, methacrylamides and mixtures thereof. Preferred hydrophobic polymers are alkyl and arylalkyl acrylamides and methacrylamides of 4 to 20 carbons, and alkyl and arylalkyl acrylates and methacrylates of 4 to 20 carbons. When the method is to be used to remove viral inactivating agents from blood and blood fractions, particularly preferred polymers are N-tert-octyl acrylamides, N-octadecyl acrylamide, N-methylundecyl acrylamide, and octadecyl methacrylate.

The method of the invention is particularly well suited to removing up to 5% by weight of one or more viral inacti-

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vating agents selected from the group consisting of detergents and hydrophobic solvents. The method is particularly useful for removing a phosphate ester such as tri-n-butyl phosphate, a detergent such as an ethoxylated nonylphenol or octylphenol nonionic surfactant, or a combination of solvent and detergent.

In another aspect the invention relates to a porous support for removing small exogenous molecules from biological fluids. The support comprises a porous mineral oxide matrix which has interior pore volume substantially filled by a cross-linked hydrophobic polymer. The hydrophobic polymer overlays but is not covalently bound to the mineral oxide matrix. The hydrophobic polymer has an exclusion limit of about 10 kilodaltons. Preferred matrices and hydrophobic polymers are as described above for the method using the porous support.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of the putative architecture of the three-dimensional polymer network formed within and extending from the internal surfaces of an individual pore in a porous solid matrix according to the present invention.

FIG. 2 is a schematic illustration of the presumed architecture of a standard octadecylsilane-coated silica matrix of the art.

#### DETAILED DESCRIPTION OF THE INVENTION INCLUDING PREFERRED EMBODIMENTS

The present invention relates to the synthesis of a special solid sorbent with a specific internal chemical structure that is able to selectively absorb small hydrophobic molecules or amphiphilic molecules, such as detergents, that have a significant hydrophobic domain and a polar domain. The sorbents comprised of two main components: (1) a rigid porous mineral material which may be capable of forming hydrogen bonds with the polar domain of amphiphilic molecules such as detergents and (2) an organic hydrophobic network within which non-polar solvents can be effectively retained. The organic hydrophobic network is highly crosslinked so that detergents, solvents and other contaminants smaller than 10,000 dalton are easily captured inside the microporous structure while proteins and cellular components slip around the particles and do not interact with the sorbent.

The sorbents of the invention allow an in-line process for the removal of hydrophobic molecules with higher throughput than is possible with oil extraction technology. The method has the additional advantage that no waste solvents are produced whereas oil extraction produces a solvent enriched oil that must be regenerated or disposed of. In addition, contamination of the biological fluid by the purification medium is avoided, whereas in the case of oil extraction the oil is likely to be adsorbed in traces by the hydrophobic proteins in the biological fluid.

The sorbents of the invention provide a number of advantages over gel filtration as well. Sample loading is a limiting factor in gel filtration. Commonly, the volume of load cannot be higher than 25% of column volume, so that a ten liter column is needed to treat about 2.5 liters of biological fluid. In contrast, a ten liter column of the sorbent of the invention could be used to treat as much as 100 liters of biological fluid. The linear flow rate of such a gel filtration column is limited to less than centimeters per hour because of the

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mechanical instability of the gel and the band broadening that occurs with higher linear velocities; by contrast the particles of the invention allow high flow rates.

The polymer filled mineral oxide sorbents of the invention show very high chemical and physical stability and are relatively unaffected by solvents, strongly acidic aqueous media, strongly alkaline media, and oxidizing agents.

Chemical and physical stability are important characteristics of a material that is to be reused a number of times and that must therefore be cleaned and regenerated. In particular, it is important that regeneration conditions not give rise to degradation products that would either impair the original properties of the sorbent or introduce contaminants into the biological fluid. In respect to stability, the sorbent of the invention provides a number of advantages over existing methodologies for the removal of solvents and detergents from biological fluids.

Chemical stability and sorption capacity are the primary drawbacks of reverse phase chromatography on C-18 silica for the removal lipid solvents. The C-18 solid phase cannot be used above pH 8 because of chemical degradation of the substrate. Moreover, C-18 reverse phase sorbents are notorious for their non-specific adsorption of lipophilic proteins from biological fluids.

The pores of the mineral oxide sorbents of the present invention are filled with a stable crosslinked polymer which is resistant to chemical breakdown. In addition, the reversible sorption capacity is higher due to the density of lipid chains in the polymer network. While the number of octadecyl hydrocarbon chains is limited on C-18 reverse phase substrates by the number of accessible hydroxyls on the surface of silica, in the present invention, it is not so limited. Because the volume of polymer is a function of pore volume rather than pore surface area, and because the polymer is not bound to the silica, a dense network can be laid down by using a more concentrated monomer for polymerization.

The mineral component of the sorbent of the invention is characterized by a high surface area per gram in order to maximize the adsorptive capacity for detergents and similar molecules having polar domains. By the same token, the amount of organic hydrophobic polymer network is high enough to allow maximum sorption of non-polar solvents. The high crosslinking, leading to an exclusion limit below 10 kilodaltons, restricts diffusion to relatively small molecules and prevents the penetration and consequent non-specific binding of proteins.

The preparation of the sorbents of the invention is similar in some respects to the preparation of sorbents described in U.S. Pat. No. 5,268,097 which is incorporated herein by reference. A solid porous mineral in bead form or in the form of irregular particles is impregnated with a solution of appropriate hydrophobic polymerizable monomers and bifunctional crosslinkers. After filling the pores of the mineral with monomer solution, polymerization is effected under the action of a catalyst. The crosslinked polymer is immobilized inside the porous volume of the mineral substrate by physical trapping and cannot escape even under the action of solvents.

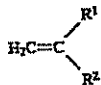
The mineral substrate can be any metal oxide that is capable of providing a porous structure and that can be obtained in irregular or bead-shaped particles. The metal oxide may additionally possess the ability to hydrogen bond with detergents possessing polar side chains. Silica, alumina, zirconia, titania and mixtures of them are examples of useful mineral materials.

Monomers useful for preparing the immobilized crosslinked hydrophobic polymer include vinylic, acrylic,

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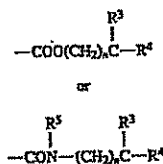
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and allylic monomers. They are characterized by the presence of a hydrophobic side chain which can be aromatic, heterocyclic or aliphatic. Aliphatic side chains can be linear, branched, or cyclic. Examples of monomers include octadecyl methacrylate, hexadecyl methacrylate, dodecyl methacrylate, octyl methacrylate, octadecyl acrylamide, hexadecyl acrylamide, methylundecyl acrylamide, iso-octyl acrylamide, hexyl acrylamide, phenylpropyl acrylamide and trityl acrylamide. Exemplary vinylic, allylic and acrylic monomers correspond to the general formula I:



wherein

$R^1$  is hydrogen or methyl and  $R^2$  is hydrogen, lower alkyl,



$n$  is zero or an integer from 1 to 18;

$R^3$  is hydrogen, alkyl or aryl;

$R^4$  is hydrogen, alkyl, aryl, or heteroaryl; and

$R^5$  is hydrogen or alkyl.

To prepare sorbents of the invention, a bifunctional crosslinking agent is added to the monomer. The crosslinking agent allows the three dimensional insoluble polymeric network to form within and substantially fill the pore volume of the porous matrix. In the absence of the crosslinker, the polymer formed would be linear and, because of its solubility, could be extracted from the pore by common solvents. The amount of crosslinking agent should be about 0.1% to about 10% by weight of the total weight of monomer. Crosslinking agents used in the present invention are acrylic, vinylic or allylic monomers that possess at least two polymerizable functional groups. Preferred crosslinking agents have at least two double bonds and are those classically used in making acrylic, vinylic and allylic polymers. Examples of useful crosslinking agents include, but are not limited to,  $N,N'$ -methylene-bis-acrylamide,  $N,N'$ -methylenebis-methacrylamide, diallyl tartradiamide, allyl methacrylate, diallyl amine, diallyl ether, diallyl carbonate, divinyl carbonate, divinyl ether, 1,4-butanedioldivinylether, and 1,3-diallyloxy-2-propanol.

After mixing the monomer and crosslinking agent, the mixture is admixed with the porous solid matrix thereby filling the pores of the matrix. In one possible process for preparing the sorbent, the pores are filled with an aqueous solution of monomer and crosslinking agent and the mixture is placed in a non-aqueous dispersing medium. Suitable non-aqueous media include non-polar organic solvents known to those skilled in the art, for example, vegetable oils, aromatic solvents and chlorinated solvents. Preferred non-aqueous media include toluene, methylene chloride and hexane.

Thereafter a polymerization initiator is added to the mixture. Examples include amines such as  $N,N,N'$ -tetramethylethylenediamine (TMEDA) or dimethylaminopropionitrile that are commonly used with oxidizing initiators (see below) such as ammonium persulfate. These may also include photoactivatable compounds such as riboflavin or

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thermoactivatable chemicals such as azo-bis-isobutyronitrile, ammonium persulfate or azo-bis-amidinopropane. The concentration of initiator is from about 0.1 to about 2%. It will be apparent to those of skill in the art that certain initiators are best dissolved in aqueous media while others are best dissolved in organic media. Hence, depending on the solubility characteristics of a particular initiator or combination of initiators, the polymerization initiator can be added to the initial solution of monomer and crosslinking agent prior to addition of that mixture to the porous solid matrix. In fact, an initiator combination of ammonium persulfate and tetramethylethylenediamine (TMEDA) can be introduced separately. The water soluble persulfate salt is combined with the aqueous mixture of monomer and crosslinking agent while the TMEDA is combined with the non-aqueous dispersing medium. It should be noted that the persulfate/TMEDA combination is particularly useful because TMEDA displays appreciable solubility in water and is thereby able to penetrate the pores of the treated support to efficiently initiate polymerization. When using the combination of persulfate and tertiary amine, the persulfate is preferably added prior to the addition of the non-aqueous medium, since persulfate is much more soluble in water than in non-aqueous dispersing media.

The polymerization process creates a three dimensional lattice or crosslinked polymer network 1 that extends away from the pore wall surfaces 2 of the porous solid matrix as illustrated in FIG. 1. The three dimensional structural lattice substantially fills the porous volume, and is substantially identical in shape to that of the pore which it fills. This is distinguished from the structure of typical coated silica (see FIG. 2) where the aliphatic residues 3 are scattered in a monomolecular layer along the surface of the silica 2.

It has been discovered that the porous supports of the present invention exhibit unusually high dynamic sorptive capacity as a function of flow rate for the removal of hydrophobic molecules. In particular, whereas a great majority of porous materials suffer a marked decrease in sorptive capacity as flow rates increase, the supports of the present invention show little decrease in useful sorptive capacity for hydrophobic molecules from a static condition up to flow rates of several hundred cm/hr. This is in marked contradistinction to the behavior of polysaccharide gel type materials such as Sepharose. Moreover, the absolute capacity of the supports of the present invention are considerably greater than those of other types of solid supports that exhibit a similar insensitivity to high flow rate. Interestingly, the sorbents appear more like typical porous supports in respect to their behavior with detergents.

The sorbents and methods of the invention can be used to remove various small exogenous molecules from biological fluids. Biological fluids of interest include, but are not limited to, blood and blood fractions, semen, milk, ascitic fluid, saliva, placental extracts, tissue culture cell lines and their extracts including transformed cells and products of fermentation.

Of primary interest among solvents to be removed are the dialkyl phosphate and trialkyl phosphate solvents having linear or branched groups from 1 to 20 carbon atoms. Examples of this group are tri-( $n$ -butyl)phosphate, tri-( $t$ -butyl)phosphate, tri-( $n$ -hexyl)phosphate, and tri-(2-ethyl-hexyl)phosphate. Lipophilic solvents include in addition to the phosphate esters mentioned above, halo hydrocarbons and ethers, which have also been used for virus inactivation.

The media are also useful in removing detergents or surfactants of all sorts. The hydrophilic domain of the detergent can be non-ionic (e.g. polyoxyethylene chains,

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mono or poly hydroxylated chains, sugars, and the like), anionic (e.g. carboxylates, sulfonic acids, sulfates, phosphates or phosphonates) or cationic (e.g. ammonium salts and pyridinium salts). The hydrophobic domain of the detergent can include alkyl, aryl or heteroaryl residues. Examples of non-ionic detergents include: (a) the polyethylene oxide condensates of alkyl and dialkyl phenols, having a straight or branched alkyl of from about 6 to about 12 carbon atoms with ethylene oxide and (b) the condensation products of aliphatic alcohols with ethylene oxide of the formula  $RO(C_2H_4O)_n$ , wherein R is a straight or branched alkyl having from about 8 to about 22 carbon atoms and n is 3 to 40. Non-ionic surfactants of type (a) are marketed by GAF Corporation under the trademark IGEPAL™ and by Union Carbide under the trademark Triton™. Of particular interest are Triton X100 and Triton X45, which have been used to inactivate viruses in blood and blood products. Non-ionic surfactants of type (b) above are marketed by Shell Chemical Company under the trademark Neodol™ and by Union Carbide Corporation under the trademark Tergitol™. Other non-ionic surfactants include polyoxyethylenated derivatives of sorbitan monolaurate, known as polysorbates; of particular interest is polysorbate 80 which has been used to inactivate virus in blood and blood products.

Anionic surfactants of interest include sodium cholate and sodium taurodeoxycholate. Cationic surfactants include cetyltrimethylammonium bromide, cetylpyridinium chloride and dodecylpyridinium chloride. Zwitterionic surfactants include phosphatidyl choline and sulfobetaines such as N-dodecyl-N,N-dimethyl-2-amino-1-ethanesulfonate. Other non-ionic detergents include amides of tris-hydroxymethylamino methane containing alkyl chains, alkyl glycosides and other lipopolysaccharides. Other species of interest include fatty acids, such as caprylic acid, and triterpenoids, such as carbenoxolone, which have also been used to inactivate virus in blood and blood products.

The elimination of undesired exogenous chemical agents is valuable for other processes besides the removal of viral inactivating agents. For example, phorbol esters, which are well known carcinogens, were used to stimulate lymphokine production and must be removed from the product before administration. By the same token, the use of detergent is not restricted to virus inactivation; detergents used for the purification of vaccine antigens must also be removed at the end of the purification process [see *Biochem. Biophys. Acta* 415, 29 (1975)].

The invention is further illustrated by the following examples:

#### EXAMPLE 1

Preparation of a silica-polymer composite with medium length aliphatic hydrophobic chain.

Four grams of methylundecylacrylamide (MUA) was dissolved in 3 mL of pure ethanol at 40°-50° C. Separately, 0.4 mg of N,N'-methylene-bis-acrylamide (MBA) was dissolved in 1.5 mL of dimethylsulfoxide. The two solutions were mixed together and 0.5 mL of demineralized water containing 0.05 mg of azobis-amidinopropane was added to the solution. The total volume of the mixture was adjusted to 10 mL with pure ethanol.

The solution of monomers was added dropwise under stirring to 10 g of dry porous silica, the surface area of which was 200 m<sup>2</sup>/g and the porous volume about 1 cm<sup>3</sup>/g. Under nitrogen, the impregnated silica was heated in a closed vessel at 80°-90° C. for at least two hours to begin the

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polymerization. The polymer-silica composite obtained was cooled overnight and then washed extensively with ethanol, 0.5M sodium hydroxide, 0.1M hydrochloric acid and finally with water.

The composite sorbent was placed in a 0.3x10 cm column and 6 mL of bovine serum treated with 5 mg/mL of TNBP and 10 mg/mL of Triton X-100 according to the method of Horowitz et al. [*Transfusion* 25, 516-522 (1985) and *Blood* 79, 826-831 (1992)] was flowed through the column. Both Triton X-100 and tri-n-butylphosphate (TNBP) were removed from the solvent/detergent virus inactivated bovine serum. The sorption capacity for Triton X-100 was about 60 mg/mL of sorbent. The sorption capacity for TNBP was greater than 43 mg/mL of sorbent.

#### EXAMPLE 2

Preparation of silica polymer composite with long aliphatic hydrophobic chains.

Two grams of octadecylacrylamide (ODA) was dissolved in 15 mL of dichloroethane under stirring. Separately 0.8 mg of N,N'-methylene-bis-methacrylamide (MBMA) was dissolved in 3 mL of methanol and mixed with the ODA solution. The resulting mixture, 2 mL of methanol containing 0.1 mg of azobis-isobutyronitrile, was added and mixed thoroughly. Ten milliliters of the monomer solution was added dropwise and under stirring to 10 g of dry porous silica, the surface area being about 200 m<sup>2</sup>/g and the porous volume about 1 cm<sup>3</sup>/g.

Under nitrogen stream, the solvents (dichloroethane and methanol) were evaporated to constant weight. On the resulting dry material, the second half of the monomer solution (10 mL) was added dropwise as described above.

Under nitrogen, the monomer-impregnated silica was heated in a closed vessel at 80°-90° C. for at least two hours to begin the polymerization. The obtained polymer-filled silica was cooled overnight and then washed extensively with dichloroethane, methanol, 0.5M sodium hydroxide, 0.1M hydrochloric acid and finally with water. It was then stored as an ethanol suspension or dried. Bovine plasma was treated as described in Example 1. The sorption capacity for Triton X-100 was 78 mg per mL of sorbent; the sorption capacity for TNBP was greater than 45 mg/mL of sorbent. Both results were obtained in the presence of bovine plasma.

#### EXAMPLE 3

Preparation of polymer-filled silica with branched aliphatic hydrophobic chains.

The preparation of this material was performed as described in Example 1, except that the main monomer was tert-octylacrylamide instead of MUA and that the bifunctional cross-linker was MBMA instead of MBA.

The properties of this material when tested as in Example 1 were as follows:

Capacity for Triton X-100: 65 mg/mL of sorbent  
Capacity for TNBP: >43 mg/mL of sorbent

#### EXAMPLE 4

Preparation of silica-polyacrylate composite material with long aliphatic chains.

The preparation of this material was performed as described in Example 2, except that the main monomer was an octadecylmethacrylate instead of octadecyl-acrylamide. The properties of this material when tested as in Example 1 were as follows:

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Capacity for Triton X-100: 72 mg/mL of sorbent  
Capacity for TNBP: >43 mg/mL of sorbent

## EXAMPLE 5

Influence of the silica surface area on the sorption capacity for Triton X-100.

Three silica-MUA sorbents were prepared according to the methodology described in Example 1. The only variable parameter was the surface area of the porous silica. The concentration of the monomer was in all cases 40% w/v. On these three sorbents, the sorption capacity for a nonionic detergent, Triton X-100, was measured in the same conditions. This was done using bovine serum containing 1% of Triton X-100. The intrinsic sorption capacities of the sorbents were as follows:

Silica Surface area (m <sup>2</sup> /g)	Sorption capacity (mg/mL)
25	11
75	22
200	60

## EXAMPLE 6

Influence of the polymer concentration on the capture efficiency for TNBP.

Three silica-MUA sorbents were prepared according to the methodology described in Example 1. The only variable parameter was the concentration of MUA monomer prior to polymerization. The specific surface area and the porous volume of silica were in all cases respectively 200 m<sup>2</sup>/g and 1 cm<sup>3</sup>/g.

On these three sorbents, the sorption capacity for TNBP was measured in the same conditions. This was done using bovine serum containing 0.5% of TNBP. Measuring the TNBP in the column effluents, it was possible to obtain a measure of capture efficiency of the sorbents. Results were as follows:

Concentration of MUA (%)	Residual amount of TNBP (ppm)
20	120-130
30	20-30
40	10-20

Total sorption capacity for TNBP was in all cases between 40 to 45 mg/mL.

## EXAMPLE 7

Repeated depletion of solvent/detergent from a virus inactivated plasma.

A silica-MUA sorbent corresponding to the description of Example 1 was packed in a column of 0.3 cm diameter and 10 cm length. The column was equilibrated by repeated washings with a phosphate buffer physiological saline solution (PBS) at a flow rate of 0.15 mL per minute. A sample of 2.5 mL of solvent/detergent treated bovine serum (content of Triton X-100 was 1%; the content of TNBP was 0.5%) was injected into the column and collected at the column outlet. The column was then washed with 10 volumes of each following solution: PBS/ethanol 50%; ethanol; ethanol-isopropanol 50%; isopropanol; ethanol; PBS/ethanol 50%. Finally it was re-equilibrated with PBS. At this point,

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a second injection of solvent/detergent treated bovine serum was done in the same conditions as described above and then the column regenerated and re-equilibrated. This cycle was repeated five times. The five column effluents were analyzed and the content of Triton X-100 and of TNBP was assayed. The results obtained were as follows:

Cycle	Initial content		Final Content	
	TNBP (mg/mL)	Triton X-100 (mg/mL)	TNBP (mg/mL)	Triton X-100 (mg/mL)
1	5	10	$12.8 \times 10^{-3}$	0.35
2	5	10	$18 \times 10^{-3}$	0.46
3	5	10	$8.6 \times 10^{-3}$	0.30
4	5	10	$12.2 \times 10^{-3}$	0.36
5	5	10	$12.9 \times 10^{-3}$	0.37

## EXAMPLE 8

Solvent/detergent elimination from a solution of immunoglobulins G.

To 10 mL of 10 mg/mL of human immunoglobulins G (IgG) in 0.15M phosphate buffered saline at physiological pH, 0.05 mL of TNBP and 0.1 mL of Triton X-100 were added. The mixture was classically treated under gentle agitation at 27° C. for 4 hours. Two and one-half milliliters of this solution were passed through a 0.7x10 cm column of silica-MUA-sorbent; the flow rate was 0.15 mL/minute. The IgG effluent was recovered and analyzed to quantify the residual amount of solvent and detergent. Results were as follows:

	Amount of chemicals	
	TNBP (mg/mL)	Triton X-100 (mg/mL)
Before depletion	5	10
After depletion	undetectable (or <0.4 ppm)	undetectable
Removal efficiency (%)	100%	100%

## EXAMPLE 9

Solvent/detergent elimination from a whole human inactivated plasma.

To 10 mL of human plasma 0.05 mL of TNBP and 0.1 mL of Triton X-100 were added. This biological fluid was then treated as described in Example 8. The results of the treatment are indicated in the table below.

	Amount of chemicals in plasma	
	TNBP	Triton X-100
Before treatment	5 mg/mL	10 mg/mL
After treatment	3.8 ppm	342 ppm
Removal efficiency	99.92%	96.58%

## EXAMPLE 10

Influence of flow rate on the solvent/detergent depletion from a biological fluid.

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This experiment was done under the same conditions as described in Example 8. Four experiments were done in parallel in order to check the influence of flow rate on the solvent/detergent depletion efficiency. Results were as follows:

Flow Rate (mL/min)	Depletion efficiency (%)					
	TNBP			TRITON		
	Before (Mg/mL)	After (mg/mL) $\times 10^{-3}$	Removal efficiency (%)	Before (mg/mL)	After (mg/mL)	Removal efficiency (%)
0.15	5	3.8	99.924	10 mg/mL	0.34	95.2
0.3	5	3.6	99.928	10 mg/mL	0.94	84.0
0.6	5	10.6	99.788	10 mg/mL	1.72	62.0
1.2	5	31.0	99.380	10 mg/mL	2.20	51.0
2.4	5	48.0	99.040	10 mg/mL	2.50	39.0

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hydrophobic polymer chosen from the group consisting of acrylates, methacrylates, acrylamides, methacrylamides and mixtures thereof.

While the invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that other changes in form and details may be made therein without departing from the spirit and scope of the invention.

We claim:

1. A method for removing contaminants from a biological fluid comprising bringing said biological fluid into contact with a cross-linked hydrophobic polymer network overlaying, but not covalently bound to a porous mineral oxide matrix, said mineral oxide matrix having interior porous volume substantially filled by said hydrophobic polymer network, said mineral oxide matrix having interior porous volume free of hydrophilic polymers, whereby hydrophobic and amphiphilic molecules of molecular weight below 10,000 daltons are simultaneously removed from said biological fluid.

2. A method according to claim 1 for removing solvents and surfactants from a biological fluid comprising bringing said biological fluid into contact with a cross-linked hydrophobic polymer network overlaying, but not covalently bound to, a porous mineral oxide matrix whereby said solvents and surfactants are removed from said fluid.

3. A method according to claim 2 wherein said biological fluid is blood, a blood fraction, or a biological extract.

4. A method according to claim 1 wherein said mineral oxide matrix has an initial average particle size of about 1 to about 2000 microns, an initial porous volume from about 0.2 to about 4 cm<sup>3</sup>/g, an initial surface area from about 1 to about 1000 m<sup>2</sup>/g, and an initial pore size from about 50 to about 6000 Angstroms.

5. A method according to claim 4 wherein said mineral oxide matrix has an initial porous volume of about 1 cm<sup>3</sup>/g and an initial surface area of about 200 m<sup>2</sup>/g.

6. A method according to claim 1 wherein said hydrophobic polymer is selected from the group consisting of acrylates, methacrylates, acrylamides, methacrylamides and mixtures thereof.

7. A method according to claim 6 wherein said hydrophobic polymer is selected from the group consisting of N-alkyl and N-arylalkylacrylamides and methacrylamides of 4 to 20 carbons.

8. A method according to claim 6 wherein said hydrophobic polymer is selected from the group consisting of alkyl and arylalkyl acrylates and methacrylates of 4 to 20 carbons.

9. A method according to claim 1 for removing viral inactivating agents from blood or a blood fraction comprising bringing said blood into contact with a cross-linked

10. A method according to claim 9 wherein said hydrophobic polymer is selected from the group consisting of N-alkyl and N-arylalkylacrylamides and methacrylamides of 4 to 20 carbons.

11. A method according to claim 10 wherein said hydrophobic polymer is selected from the group consisting of N-tert-octylacrylamide, N-octadecylacrylamide, and N-methylundecylacrylamide.

12. A method according to claim 9 wherein said hydrophobic polymer is selected from the group consisting of alkyl and arylalkyl acrylates and methacrylates of 4 to 20 carbons.

13. A method according to claim 12 wherein said hydrophobic polymer is octadecyl methacrylate.

14. A method according to claim 9 wherein said blood contains up to 5% of one or more viral inactivating agents selected from the group consisting of detergents and hydrophobic solvents.

15. A method according to claim 14 wherein said viral inactivating agent is a phosphate ester, a detergent, or a combination of the two.

16. A method according to claim 15 wherein said viral inactivating agent is a combination of tri-n-butylphosphate and an ethoxylated alkylphenol nonionic surfactant.

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## United States Patent [19]

Sarno et al.

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[54] PROCESS FOR PURIFYING IMMUNE  
SERUM GLOBULINS

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530/418; 530/419; 530/420; 530/421

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424/85.8

## [56] References Cited

## U.S. PATENT DOCUMENTS

3,869,436	3/1975	Falksveden .....	260/122
3,962,421	6/1976	Neurath .....	424/89
4,334,997	2/1982	Shanbrom .....	424/101
4,315,919	2/1982	Shanbrom .....	424/177
4,540,573	9/1985	Neurath et al. ....	530/381
4,606,825	8/1986	Crane et al. ....	210/635
4,683,294	7/1987	Van Wijnendaele et al. ....	530/371
4,719,290	1/1988	Curry et al. ....	530/387
4,877,866	10/1989	Rudnick et al. ....	530/387

## OTHER PUBLICATIONS.

Garcia and Ornez. "The Use of Pluronic Polyols in the Precipitation of Plasma Proteins and Its Application in the Preparation of Plasma Derivatives". *Transfusion* 16:32-34 (1976).

Sober and Peterson. "Protein Chromatography on Ion Exchange Cellulose". *Fed. Proc.* 17:1116-1126 (1958).

Polson et al. "The Fractionation of Protein Mixtures by Linear Polymers of High Molecular Weight". *Biochemica et Biophysica Acta* 82463-475 (1964).

Tayot et al. "Ion Exchange and Affinity Chromatography on Silica Derivatives" in *Methods of Plasma Fractionation* (Curling, Academic Press, London) pp. 149-160 (1980).

Zolton et al. "Removal of Hepatitis B Virus Infectivity from Human Gamma-Globulin Prepared by Ion-Exchange Chromatography", *Vox Sang* 49:381-389 (1985).

Friesen et al., "Column Ion Exchange Use", *Vox Sang* 48:201-212 (1985).

Webb, "A 30-Minute Preparative Method for Isolation of IgG from Human Serum", *Vox Sang* 23:279-290 (1972).

Stanworth, "A Rapid Method of Preparing Pure Serum Gamma-Globulin", *Nature* 488:156-157 (1960).

Bjorling, "Plasma Fractionation Methods Used in Sweden", *Vox Sang* 23:18-25 (1972).

Cohn et al., *J. Am. Chem. Soc.* 68:459-475 (1946).

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## [57] ABSTRACT

A multi-step process for purifying an immune serum globulin fraction from a crude plasma protein fraction involves precipitating non-serum globulin proteins from an aqueous suspension of the crude plasma protein fraction using a protein precipitant, adding a virus-inactivating agent to the clarified immune serum globulin-containing liquid, absorbing the immune serum globulins onto a cation exchange resin and washing non-serum globulin contaminants from the resin, subjecting the eluate to ultrafiltration to concentrate the immune serum globulins and separate them from low molecular weight species, contacting the concentrate with an anion exchange resin to absorb non-serum globulin contaminants, passing the immune-serum globulins through the anion exchange resin under conditions that leave non-serum globulin contaminants bound to the resin, and subjecting the filtrate to a molecular washing step to produce a purified immune serum globulin fraction. This process results in products substantially free of active viruses and contaminating lipids, activated complements and low molecular weight peptides. In addition, the process is advantageous in terms of efficiency and adaptability to large-scale production.

20 Claims, No Drawings

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## PROCESS FOR PURIFYING IMMUNE SERUM GLOBULINS

### BACKGROUND OF THE INVENTION

This invention relates to a process for purifying immune serum globulins. More particularly, the invention relates to a process for purifying immune serum globulins from a crude plasma protein fraction.

Blood plasma proteins serve a wide variety of functions in the mammalian body. These proteins are involved in the maintenance of blood volume, viscosity, osmotic pressure, and other important physical parameters. Certain plasma proteins are themselves important biologically active molecules or act as carriers for vital nonprotein molecules. A large group of plasma proteins is concerned with the immune response. The immune serum globulins, also known as gamma globulins, include antibodies directed against many disease causative agents.

Fractionation of human plasma has long been used to produce therapeutic materials containing one or more of the plasma proteins in concentrated and purified form to achieve optimal clinical usefulness. Various fractionation schemes have been employed for recovering clinically useful proteins from human plasma. One scheme in widespread use is the well-known Cohn fractionation method, which is based on differential precipitation using cold ethanol. Cohn et al., *J. Am. Chem. Soc.*, 68, 459 (1946).

The Cohn fractionation procedure initially produces crude plasma protein fractions, which are subsequently refined to purified products.

A need exists for an efficient process for purifying an immune serum globulin fraction from a crude plasma protein fraction. Such a process should be amenable to large scale production and should inactivate any blood-carried viruses which might be present in the crude plasma fraction.

### SUMMARY OF THE INVENTION

In accordance with the present invention, a process for purifying an immune serum globulin fraction from a crude plasma protein fraction is provided. This process involves the steps of: suspending the crude plasma protein fraction in water and precipitating a major proportion of the non-serum globulin proteins with a protein precipitant, recovering a clarified immune serum globulin-containing solution, adding a virus-inactivating agent to the clarified immune serum globulin-containing solution, adsorbing the immune serum globulins onto a cation exchange resin and washing non-serum globulin contaminants from the resin, eluting the immune serum globulins from the cation exchange resin and subjecting the eluate to ultra-filtration to concentrate the immune serum globulins and separate them from lower molecular weight species, contacting the concentrate with an anion exchange resin to absorb non-serum globulin contaminants, passing the immune-serum globulins through the anion exchange resin under conditions that leave non-serum globulin contaminants bound to the resin, and subjecting the filtrate to a molecular washing step to produce a purified immune serum globulin fraction.

The starting material for this process can be any crude plasma protein fraction which contains the immune serum globulins. A preferred starting material is the Cohn Fraction I+II+III. This process has been found particularly advantageous in terms of efficiency

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and adaptability to large-scale production. In addition, the process results in a product substantially free of active viruses and substantially free of contaminating lipids, activated complements (e.g., C5a, C3a, and the like) and low molecular weight peptides which may have adverse physiological affects.

### DETAILED DESCRIPTION OF THE INVENTION

The starting material for the present process advantageously is an immune serum globulin-containing fraction from a conventional plasma fractionation process. A particularly preferred crude plasma protein fraction is the Fraction I+II+III precipitate from a large-scale Cohn fractionation procedure. This crude plasma protein fraction is usually obtained by subjecting a conventional cryoprecipitate supernatant to cold ethanol precipitation at pH 6.9. In addition to the immune serum globulins, Cohn Fraction I+II+III contains fibrinogen, various lipoproteins, several proteins involved in the hemostatic and fibrinolytic systems and numerous minor components. Although Cohn Fraction I+II+III is a preferred starting material, other starting materials may be used for the present process. Such materials include, for example, plasma, cryoprecipitate-free plasma, Cohn Fraction II+III and Cohn Fraction II.

The first step of the process involves suspending the crude plasma protein fraction in water at a substantially non-denaturing temperature and acidic pH. As used herein, "substantially non-denaturing" means that the condition to which the term refers does not cause substantial irreversible loss of biological activity of the immune serum globulins. Advantageously, the crude plasma protein fraction is suspended in cold water at volumes 5 to 10 times the weight of the fraction. The water is preferably maintained at a cold temperature which prevents substantial denaturation of the immune serum globulin proteins. Temperatures of from about 0° to about 10° C., preferably from about 1° to about 3° C., are typically employed. The suspension is acidified with a non-denaturing acid. The pH of the suspension preferably is maintained from about 4.5 to about 5.5, preferably from about 5.0 to about 5.2.

Non-serum globulin proteins are precipitated from the suspension using a protein precipitant. Substantially non-denaturing, water soluble protein precipitants are well-known in the protein purification arts. Such precipitants are used for the differential precipitation, and thus partial purification, of proteins from aqueous solutions or suspensions. Suitable protein precipitants for use in the process of the present invention include various molecular weight forms of polyethylene glycol, ammonium sulfate, polyvinylpyrrolidone and pluronics. Several grades of pluronic polyols (Pluronic® manufactured by the BASF Wyandotte Chemical Corporation) are effective protein precipitants. These polyols, of diversified molecular weight (from 1,000 to over 16,000) and physicochemical properties, have been used as surfactants. A family of 32 polyols with a variety of liquid, paste and solid forms is available. Pluronic F-38, of a molecular weight of 5,000, and Pluronic F-68, of molecular weight 9,000, both contain (by weight) 80 per cent hydrophilic polyoxyethylene groups and 20 per cent of hydrophobic polyoxypropylene groups. Polyethylene glycol is a preferred precipitant, particularly polyethylene glycol 3350 (PEG 3350) or polyethylene

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glycol 6000 (PEG 6000) (numbers represent average molecular weight of the compound).

The protein precipitant is added to the aqueous suspension in an amount sufficient to cause precipitation of a major proportion of contaminating proteins, lipids and some viruses, without causing substantial precipitation of immune serum globulins. The protein precipitant may be added to the crude plasma protein suspension as a solid, or an aqueous concentrate derived from the commercially available solid powder or flakes. The actual amount of protein precipitant used will vary, depending upon the particular precipitant employed, the temperature, pH and protein concentration in the suspension. When PEG 3350 is used, final concentrations of the precipitant in the aqueous suspension advantageously range from about 3% to about 20% by weight, preferably from about 6% to about 12% by weight. The precipitation is allowed to proceed until equilibrium is reached, e.g., generally for about one hour or more. The suspension preferably is maintained at a low temperature (e.g., less than about 10° C., preferably less than about 5° C.) throughout the precipitation step.

Following precipitation, a clarified immune globulin-containing liquid is recovered from the solids-liquid mixture resulting from the precipitation. Recovery of the clarified liquid can be accomplished by conventional solids-liquid separation techniques, such as centrifugation and filtration. Preferably, a centrifuge with at least about 5,000 G force or a tangential flow filter system with micro filtration membranes is employed.

Infectious viruses that may still be present in the crude plasma protein fraction can be inactivated at this stage of the procedure. Such inactivation is accomplished by adding a virucidal amount of a virus-inactivating agent to the clarified immune serum globulin-containing liquid. Preferred virus-inactivating agents are detergents, most preferably, detergent-solvent mixtures. A wide variety of detergents can be used for virus inactivation. Suitable detergents are described, for example, by Shanbrom et al., in U.S. Pat. Nos. 4,314,997, 4,315,919, and 4,540,573, the disclosures of which are incorporated herein by reference. Preferred detergents are oxyethylated alkylphenols, such as those sold by the Rohm & Haas Company under the trademark, Triton X-100, and polyoxyethylated derivatives of a partial ester of a C<sub>12-22</sub> fatty acid and a hexatol anhydride, such as those sold under the trademark Tween 80. Preferred solvents for use in virus-inactivating agents are the lower alkyl esters of phosphoric acid, as described, for example, by Neurath in U.S. Pat. No. 3,962,421, the disclosure of which is incorporated herein by reference. A particularly preferred solvent is tri(n-butyl) phosphate. A preferred virus-inactivating agent for the practice of the present invention is a mixture of tri(n-butyl) phosphate, Triton X-100 and Tween 80. The mixture is formulated and used such that the concentration of tri(n-butyl) phosphate in the clarified immune serum globulin-containing liquid ranges from about 0.2 to about 0.4% by weight, the concentration of the Triton X-100 ranges from about 0.7 to about 1.3% by weight, and the concentration of the Tween 80 ranges from about 0.2 to about 0.4% by weight.

The virus-inactivating step is conducted under virus-inactivating conditions. In general, such conditions include a temperature of from about 10° C. to about 30° C., preferably from about 18° C. to about 22° C., and an incubation time found to be effective by experimenta-

tion. Generally, an incubation time of about one hour is sufficient.

After virus inactivation, the solution is contacted with a cation exchange resin to remove the virus-inactivating agent and other non-serum globulin contaminants. This step is preferably conducted by passing the solution over a column packed with a cation exchange resin, such as carboxymethyl agarose. The column preferably is equilibrated with a buffer which converts the resin to the salt form. A preferred buffer is an acetate buffer having an acetate concentration ranging from about 5 to about 50 millimolar, preferably from about 10 to about 20 millimolar. Suitable acetate buffers may be prepared from sodium acetate trihydrate and glacial acetic acid, and have a pH ranging from about 5 to 6. Another preferred buffer is a phosphate buffer having a pH of 5 to 6.

Prior to loading the immune serum globulin-containing liquid onto the column, the salt concentration of that liquid preferably is adjusted to an amount substantially equivalent to the salt concentration of the equilibration buffer. For example, if an acetate buffer is used for the acetate concentration in the immune serum globulin-containing liquid is adjusted to approximately the same concentration as that in the buffer. After loading the immune serum globulin-containing liquid onto the column, the column is advantageously washed sequentially with the same buffer used for equilibration. A preferred procedure involves employing sequential washes with decreasing concentrations of the virus-inactivating agents, with a final wash of at least ten times the bed volume of the column with a buffer devoid of the virus-inactivating agent. Sequential washes are advantageous in reducing resin-bound lipids while also removing the virus-inactivating agents from the cation exchange resin. Sequential washing has also been found to reduce pre-kallikrein activator, thus resulting in a final product substantially free of this protein. For example, after loading the column, it is washed with at least two times its bed volume with a 10 mM acetate buffer, pH 5.0-6.0, containing 1% Triton X-100 and 0.3% Tween 80, or a 10 mM acetate buffer, pH 5.0-6.0, containing 1% Triton X-100. This washing may be followed by washing with at least four times the column bed volume with a 10 mM acetate buffer, pH 5.0-6.0, containing 1% Tween 80 until the absorbance at 280 nm is less than about 1.2. When the A<sub>280</sub> has decreased below 1.2, the column advantageously is washed with at least 20 times its bed volume with 10 mM acetate buffer, pH 5.0-6.0.

The immune serum globulins are eluted from the cation exchange resin with a substantially non-denaturing buffer having a pH and ionic strength sufficient to cause substantial elution of the immune serum globulins. In general, the pH of the eluting buffer is in the basic range, preferably from about 7.0 to about 8.5. The salt concentration of the eluting buffer is relatively high to displace the immune serum globulin proteins from the resin. A preferred buffer contains about 25 mM tris(hydroxymethyl)aminomethane, about 0.25M sodium chloride, about 0.1% polyethylene glycol and about 0.2M glycine at pH 8.0. The polyethylene glycol and glycine combination serves to stabilize the protein during the elution step. Numerous other buffer systems may be used for eluting the immune serum globulins, as will be appreciated by those skilled in the art.

Following elution from the cation exchange column, the eluate is advantageously concentrated by ultrafiltra-

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tion. The extent of concentration may vary considerably. Concentrating the solution to about 1 to about 3% by weight protein, preferably from about 1.5 to about 2.5% protein has been found satisfactory. The ultrafiltration membranes employed advantageously have a molecular weight cut-off ranging from about 10,000 to about 100,000. A particularly preferred membrane for the present process is a PTHK polysulfone membrane with a nominal molecular weight cut-off of 100,000, obtained from Millipore Corp. Other commercially available ultrafiltration membranes of comparable porosity may be employed. Following concentration, the concentrate is advantageously molecular washed using the same ultrafiltration system. This step effectively removes low molecular weight peptide contaminants and provides a means for buffer exchange required for the next purification step. A preferred solution for the molecular washing step is an aqueous solution containing from about 0.005 to about 0.012% by weight polyethylene glycol. The polyethylene glycol serves to stabilize the protein.

The molecular washing is continued until the salt concentration of the ultrafiltrate is reduced to a point that the solution conductivity of less than about 5 mMH0/cm, preferably less than about 3 mMH0/cm.

The pH of the concentrated solution is adjusted to a substantially non-denaturing basic pH, e.g., from about 7.0 to about 8.5. The concentrate is then contacted with an anion exchange resin to absorb non-serum globulin contaminants. This step is advantageously conducted by passing the concentrate over a column packed with an anionic exchange resin, such as diethylaminoethyl-sepharose ("DEAE-SEPHAROSE"). The anion exchange column is first equilibrated with a basic buffer which converts it to the chloride form. Any of a variety of buffers can be employed, and a preferred buffer is 25 mM tris(hydroxymethyl)aminomethane, 20 mM sodium chloride, pH 8.0. Those skilled in the art will appreciate that numerous other buffers may be used for equilibration. Prior to loading the immune serum globulin concentrate onto the resin, it may be prefiltered to ensure that the solution is free of particulate matter.

The immune serum globulin concentrate is loaded onto the column. Most of the serum immune globulins flow unadsorbed through the column and effective recovery is accomplished by washing with at least two times the bed volume of the same buffer used for equilibration. The immune serum globulin containing fractions are collected and combined and the pH is adjusted to a substantially non-denaturing acidic pH. The purified immune serum globulin solution is again concentrated by an ultrafiltration step, which also removes salts and low molecular weight contaminating species. Additional molecular washing steps may be performed substantially as described above. These steps result in a highly purified immune serum globulin fraction.

The process of this invention offers a number of advantages over processes described in the prior art. It is a relatively fast procedure and avoids the need of further purifying crude plasma protein fractions, such as Cohn Fraction I+II+III to Cohn Fraction II for use as starting material for obtaining therapeutic products. In addition, the process is efficient in terms of labor and yield. For example, it usually takes 4-5 days to process Cohn Fraction I+II+III to Cohn Fraction II using the Cohn process. An additional 2-3 days are required to purify Cohn Fraction II to an acceptable product. Using the process of the present invention, a high qual-

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ity immune serum globulin product can be obtained in 3-4 days. Moreover, the product resulting from this process has reduced low molecular weight peptides which may have adverse physiological effects. The present process also provides better immune globulin yields than conventional procedures starting with Cohn Fraction II. Another advantage of the process is its amenability to large scale production.

A particular advantage of the process is that it removes components of the crude plasma protein fraction which might be deleterious to patients receiving therapeutic amounts of the immune serum globulin fraction. Viruses such as Hepatitis B and HIV are inactivated. Contaminating lipids and activated complement components, such as C5a, C3a, etc., are also reduced to very low levels.

The invention is further illustrated by the following examples, which are not intended to be limiting.

#### EXAMPLE

Cohn Fraction I+II+III was suspended in ten (10) volumes of water at about 2° C. The pH was adjusted to about 5.0 with 1M acetic acid. After the pH stabilized, a 50% polyethylene glycol 3350 ("PEG 3350") solution was added to the aqueous suspension in an amount sufficient to produce a PEG 3350 concentration of 8% in the resulting suspension. The suspension was mixed completely and precipitation was permitted to proceed for one hour at a temperature of about 2° C. The resulting precipitate and undissolved paste was separated by centrifugation at 5000 g force or by filtration. The precipitate was discarded and the supernate was clarified by filtration. The clarified solution was then brought to ambient temperature (22° C.) prior to the addition of detergents. Solvent/detergent was added to the solution in an amount sufficient to result in a final concentration by weight of solution of 1% Triton X-100, 0.3% Tween-80, and 0.3% tri-n-butyl-phosphate (TNBP). The detergent-treated solution was permitted to incubate for one (1) hour at 22° C. to permit viral inactivation.

After the incubation period, the acetate concentration of the treated solution was adjusted to 10 mM. The detergent and some protein contaminants were separated from the immune serum globulin by absorption of the latter to a cation exchange resin. The acetate-adjusted, detergent-treated solution was loaded onto a CM-Sepharose Fast Flow column which had been previously equilibrated with 10 mM acetate buffer solution, pH 5.5. Detergent and protein impurities were washed from the protein-bound resin with 2x the bed volume of a 10 mM acetate buffer, pH 5.5, containing 1% by weight Triton X-100 and 0.3% by weight Tween-80. This was followed by washing with 4x the bed volume with 10 mM acetate buffer, pH 5.5, containing 1% by weight Tween-80, until the optical density at 280 nm. of the spent wash solution was less than 1.2. This was then followed by washing with 20x the bed volume with 10 mM acetate buffer, pH 5.5.

Following the washing steps, the bound immune serum globulin was eluted from CM-Sepharose Fast Flow column with a buffer solution of 25 mM tris(hydroxymethyl)aminomethane ("tris"), 0.25M NaCl, 0.1% by weight PEG 3350, and 0.2 M glycine at pH 8.0. The pH of the eluate was adjusted to 5.2. Then the eluate was concentrated to approximately 2% by weight of protein using an ultrafiltration ("UF") system with PTHK membranes (polysulfone membranes with

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nominal molecular weight cut-off of 100,000). The concentrate was molecular washed and diafiltered using the same UF system with 0.008% by weight PEG 3350, until the conductivity was below 2 mMHΩ/cm. The pH of the solution was adjusted to 8.0 with 2 M tris. pH 8.4, and the clarified solution was adsorbed through an anionic DEAE-Sepharose Fast Flow column, which had been previously equilibrated with a buffer containing 25 mM tris and 20 mM NaCl at pH 8.0. The void-volume of non-adsorbed solution (DEAE-filtrate) containing immune serum globulin was collected. To recover entrapped immune serum globulin, the DEAE column was then washed with 2X the bed volume with the same buffer as previously used for equilibration, containing 25 mM tris, 20 mM NaCl at pH 8.0. The postwash was added to the DEAE filtrate. The pH was adjusted to 5.2 with 1M citric acid or equivalent and diafiltered with a 0.005% PEG solution if necessary until low molecular weight activated complement proteins could no longer be detected using conventional RIA methods. The glycine concentration of the solution was adjusted to 0.2 M and made isotonic with sodium citrate. The solution was then concentrated to 10%. The 10% solution was stabilized with a final concentration of 0.007% Tween-80, sterile filtered, and filled into appropriate containers.

We claim:

1. A process for purifying an immune serum globulin fraction from a crude plasma protein fraction, which comprises the steps of:

- (a) providing an aqueous suspension of a crude plasma protein fraction at a substantially non-denaturing temperature and acidic pH, wherein the protein concentration in the aqueous suspension is sufficient that, during the following precipitation step, non-serum globulin proteins precipitate while retaining immune serum globulins in said suspension;
- (b) adding a water soluble, substantially non-denaturing protein precipitant to the aqueous suspension of step (a) at a concentration sufficient to cause precipitation of non-serum globulin proteins, while retaining immune serum globulins in said suspension, thereby forming a solid-liquid mixture;
- (c) recovering a clarified immune serum globulin-containing liquid from the solid-liquid mixture of step (b);
- (d) adding a virucidal amount of a virus-inactivating agent to the clarified immune serum globulin-containing liquid of step (c) so as to inactivate any viruses therein;
- (e) contacting the virus-inactivated immune serum globulin-containing liquid with a cation exchange resin and washing non-serum globulin contaminants from the resin with a buffer having a pH and ionic strength sufficient to eliminate the virus-inactivating agent and other non-serum globulin contaminants from the resin while retaining immune serum globulins in said liquid;
- (f) eluting immune serum globulins from the cation resin with a substantially non-denaturing buffer having a pH and ionic strength sufficient to cause immune serum globulin elution, thereby forming an immune serum globulin-containing eluate;
- (g) subjecting the immune serum globulin-containing eluate to ultrafiltrations for concentrating immune serum globulins from said eluate and separating them from species having lower molecular

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weights, thereby forming an immune serum globulin concentrate;

(h) adjusting the pH of the immune serum globulin concentrate to a substantially non-denaturing basic pH, thereby forming a basic immune serum globulin concentrate;

(i) contacting the basic immune serum globulin concentrate with an anion exchange resin to bind the contaminating proteins as a means of separating said contaminating protein from the unbound immune serum globulin, thereby forming an immune serum globulin enriched solution; and

(j) adjusting the pH of the immune serum globulin enriched solution to a non-denaturing acidic pH and molecular washing the acidified solution using an ultrafiltration membrane which retains immune serum globulins and which possess contaminating species having molecular weights lower than those of the immune serum globulins, thereby producing a purified immune serum globulin fraction.

2. The process of claim 1, wherein the suspension in step (a) contains from about 5 to about 10 parts by volume water per part by weight of the crude plasma protein fraction, wherein the temperature of the aqueous suspension is maintained from about 0° to about 5° C., and the pH of the aqueous solution is maintained from about 4.5 to about 5.5.

3. The process of claim 2, wherein the protein precipitant employed in step (b) is polyethylene glycol, ammonium sulfate, polyvinylpyrrolidone or pluronics.

4. The process of claim 3, wherein the protein precipitant is PEG 3350 or PEG 6000.

5. The process of claim 3, wherein the virus-inactivating agent is a detergent.

6. The process of claim 3, wherein the virus-inactivating agent is a mixture of a non-denaturing detergent and a tri(lower alkyl) phosphate solvent.

7. The process of claim 6, wherein the detergent is selected from the group consisting of non-ionic, cationic and anionic detergents.

8. The process of claim 3, wherein the virus-inactivating agent is a mixture of tri(n-butyl)phosphate, an oxyethylated alkylphenol, and a polyoxyethylated derivative of a partial ester of a C<sub>12</sub>-C<sub>22</sub> fatty acid and a hexitol anhydride, wherein the concentration of the tri(n-butyl)phosphate in the clarified immune serum globulin-containing liquid is from about 0.1 to about 0.5% by weight, the concentration of the oxyethylated alkylphenol in the clarified immune serum globulin-containing liquid is from about 0.5 to about 2% by weight and the concentration of the polyoxyethylated derivative of a partial ester of a C<sub>12</sub>-C<sub>22</sub> fatty acid and a hexitol anhydride in the clarified immune serum globulin-containing liquid is from about 0.1 to about 0.5% by weight.

9. The process of claim 8, wherein the concentration of the tri(n-butyl)phosphate in the clarified immune serum globulin-containing liquid is from about 0.2 to about 0.4% by weight, the concentration of the oxyethylated alkylphenol in the clarified immune serum globulin-containing liquid is from about 0.7 to about 1.3% by weight and the concentration of the polyoxyethylated derivative of a partial ester of a C<sub>12</sub>-C<sub>22</sub> fatty acid and a hexitol anhydride in the clarified immune serum globulin-containing liquid is from about 0.2 to about 0.4% by weight.

10. The process of claim 6, wherein the cation exchange resin contains carboxymethyl groups.

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11. The process of claim 10, wherein the non-serum globulin contaminants are eluted in step (e) with an acetate buffer having an acetate concentration of from about 5 to about 50 millimolar.

12. The process of claim 10, wherein the non-serum globulin contaminants are eluted in step (e) sequentially with an acetate buffer having an acetate concentration of from about 5 to about 50 millimolar acetate, a pH from about 5.0 to about 6.0 and decreasing concentrations of the virus-inactivating agent, with a final wash of at least about 10 times the bed volume of the cation exchange resin with said acetate buffer which is devoid of the virus-inactivating agent.

13. The process of claim 10, wherein immune serum globulins are eluted from the cation exchange resin with a buffer solution having a pH of from about 7.0 to about 8.5.

14. The process of claim 13, wherein the buffer solution contains tris(hydroxymethyl)aminomethane at a concentration of from about 20 to about 30 millimolar, sodium chloride at a concentration of from about 0.2 to about 0.3 molar, from about 0.05 to about 0.2% polyethylene glycol and from about 0.1 to about 0.3M glycine.

15. The process of claim 1, wherein the ultrafiltration of step (g) is conducted with a membrane having a

molecular weight cut-off of from about 10,000 to about 100,000.

16. The process of claim 15, which further comprises molecular washing the immune serum globulin-containing eluate with an aqueous solution which contains from about 0.001 to about 0.012% by weight polyethylene glycol until the conductivity of the ultrafiltrate is at least as low as about 2 mMHO/cm.

17. The process of claim 1, wherein the anion exchange resin contains diethylaminoethyl groups.

18. The process of claim 17, wherein unbound immune serum globulins are passed through the anion exchange resin with a buffer solution having a pH of from about 7.0 to about 8.5.

19. The process of claim 18, wherein the buffer solution contains tris(hydroxymethyl)aminomethane at a concentration of from about 20 to about 30 millimolar and sodium chloride at a concentration of from about 10 to about 30 millimolar.

20. The process of claim 1, wherein, in step (j), the pH of the purified immune serum globulin solution is adjusted to about 4.5 to about 6.0, and the molecular washing is conducted with an ultrafiltration membrane having a molecular weight cut-off of from about 10,000 to about 120,000.

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UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. : 5,177,194  
DATED : January 5, 1993  
INVENTOR(S) : Maria E. C. Sarno et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On cover page, item [75] change "Maria E. Sarno" to  
--Maria E. C. Sarno--.

Col. 9, line 20, "hydroxymethyl-)" should be  
--hydroxymethyl)--.

Signed and Sealed this  
Eighth Day of November, 1994

Attest:



BRUCE LEBMAN

Attesting Officer

Commissioner of Patents and Trademarks